HDAC4 INTEGRATES PTH AND SYMPATHETIC SIGNALING IN OSTEOBLASTS

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ABSTRACT

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Both parathyroid hormone (PTH) and the sympathetic tone promote *Rankl* expression in osteoblasts and osteoclast differentiation by enhancing cAMP production, through an unidentified transcription factor for PTH and ATF4 for the sympathetic tone. How two extracellular cues using the same second messenger in the same cell elicit different transcriptional events is unknown. Here we show that PTH favors *Rankl* expression by triggering the ubiquitination of HDAC4, a class II histone deacetylase, partly via Smurf2. HDAC4 degradation releases MEF2c that transactivates the *Rankl* promoter. On the other hand, sympathetic signaling in osteoblasts favors the accumulation of HDAC4 and its association with ATF4. In this setting, HDAC4 increases *Rankl* expression. Through this interaction with ATF4, HDAC4 also influences *Osteocalcin* expression, and its endocrine and cognitive functions. This study shows that through its ability to differently connect distinct extracellular cues to their genome, HDAC4 is a global regulator of osteoblast functions.

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LIST OF ABBREVIATIONS

All abbreviations are listed in alphabetical order. In general, gene and RNA names are written in italicized format with the first letter capitalized (e.g. *Hdac4* gene or RNA) and the proteins are written in non-italicized capital letters (e.g. HDAC4 protein).

αI(1) collagen- Type 1 alpha I collagen

AC- Adenylyl cyclase

Adrβ$_2$- Adrenergic beta 2

Ag- Agamous

ALP- Alkaline phosphatase

ATF4- Activating transcription factor 4

ATPase- Adenosine triphosphatase

BAT- Brown adipose tissue

BSP- Bone sialoprotein

cAMP- Cyclic- adenosine monophosphate

CBP- CREB-binding protein

CCD- Cleidocranial dysplasia

ChIP-Chromatin immunoprecipitation

CpG- C-phosphate-G

CREB- Cyclic AMP response element binding protein

CtBP- C-terminal binding protein

CTx- C-terminal telopeptides of collagen

DAG- Diacyl glycerol

Def A- Deficiens

Dnmt – DNA Methyltransferase

Dpd- Deoxypyridinoline

ECM- Extracellular matrix

ELISA- Enzyme-linked immunoassay

Esp- Embryonic stem cell phosphatase

FGF23- Fibroblast growth factor 23

GAPDH- Glyceraldehyde 3-phosphate dehydrogenase

GLA- Gamma carboxyglutamic

GPCR- G-protein coupled receptor

GSIS- Glucose stimulated insulin secretion test

HAT- Histone Acetyltransferase

HDAC- Histone deacetylase

IP- Intraperitoneal

IP_3- 1,4,5-inositol triphosphate

ISO- Isoproterenol

ITT- Insulin tolerance test

Kb- Kilobases

M-CSF- Macrophage colony stimulating factor

MADS- **M**CM1, **A**GAMOUS, **D**EFICIENS, **S**RF

MCK- Muscle creatinine kinase

Mcm1- Minichromosome maintenance gene

MEF2- Myocyte enhancer factor 2

MITR- MEF2-intreacting transcription repressor

MWMT- Morris Water Maze test

NAD^+- Nicotine adenine dinucleotide

NES- Nuclear export signal

NF-κB- Nuclear Factor kappa B

NLS- Nuclear localization signal

OCN- Osteocalcin

OSTR-Osteocalcin receptor (Gpcr6a)

OPG-Osteoprotegerin

PKA- Protein kinase A

PKC- Protein kinase C

PTH- Parathyroid hormone

PTH1R- Parathyroid hormone receptor 1

PTHrP- Parathyroid hormone-related peptide

qPCR- Quantitative polymerase chain reaction

RANKL- Receptor activator of nuclear factor kappa-B ligand

Runx2- Runt-related transcription factor 2

SRF- Serum response factor

TSA- Trichostatin A

To my parents with love…

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CHAPTER I. GENERAL INTRODUCTION

1. Global Regulation Of Gene Expression By Modifications Of Chromatin Structure

Cell to cell differences in eukaryotes are determined by the expression of different sets of genes. The bases of these differences, in part, lay in the organization of the genetic material into a structure called chromatin. Chromatin contains the genetic information wrapped around a complex of histone and non-histone proteins. This basic repeating unit of chromatin is called the nucleosome. The nucleosome consists of an octamer of core proteins that includes 4 histone partners; an H3-H4 tetramer and two H2A-H2B dimers, which are wrapped with two superhelical turns of DNA. Histones are small basic proteins with a globular domain and a flexible and charged NH_2-terminus (histone tail) that protrudes from the nucleosome and that is subjected to post-translational modifications (Kornberg 1974; Oudet et al. 1975).

The structure of chromatin has been central to the thinking that it plays an important regulatory role and that multiple signaling pathways converge on histones. In 1928, E. Hertz discovered that there are two forms of chromatin that differs in their degree of condensation, the heterochromatin and the euchromatin (Passarge 1979). In general, many inactive genes lay in the heterochromatin (Grewal and Jia 2007). There are two types of heterochromatin; the constitutive heterochromatin, which is silenced and highly condensed, and the facultative heterochromatin, which could be regulated by cellular signals (Birchler et al. 2000; Trojer and Reinberg 2007).

On the other hand, euchromatin, the relaxed form of chromatin that allows transcriptional activity, contains many genes. The relaxed structure of the euchromatin is due to two post-translational modifications of the histone proteins, methylation and acetylation (Grunstein 1997; Santos-Rosa et al. 2002). These modifications allow specific cellular machinery, such as RNA polymerase 2 to enter the structure and transcribe functional mRNA. The presence of these two forms of chromatin was the first evidence showing the importance of the organization and the modification of chromatin in the regulation of genes.

i. Modifications of Chromatin Structure

In order to render the genetic information available, eukaryotes have developed mechanisms to modulate the structure of their chromatin. One of the modifications of chromatin structure is via DNA methylation.

DNA methylation is a covalent modification of the DNA. Prokaryotes use DNA methylation on cytosine or adenosine bases to protect the DNA from mutations that could occur during replication (Bestor 1990). Instead in eukaryotes, methylation is specific to the cytosine bases in the context of CpG islands that are distributed across the genome, with the exception of certain regions (Bird 1986). In general, methylation of these regions by DNA methyltransferases (Dnmts) in plants and mammals results in the repression of the transcriptional activity. For example, inactivation of the X chromosome is due to methylation of certain CpG islands, creating a global repression of this chromosome (Bestor 1990; Goll and Bestor 2005).

Aside from DNA methylation, the post-translational modifications of the histone tails also play a large role in regulating chromatin structure. These modifications of the histone tails include:

i. Methylation on Lysine or Arginine residues

ii. Acetylation and Deacetylation

iii. Phosphorylation

iv. Ubiquitination

Given that the work described in this thesis focuses on a class of histone deacetylases (HDACs), the next sections will discuss in detail the regulation of gene expression by histone acetylation and deacetylation.

ii. Histone acetylation and deacetylation

One of the first modifications of histones reported, acetylation, was discovered almost 60 years ago. Acetylation is a reversible post-translational modification characterized by the addition of acetyl groups to the lysines at the histone-tails. Histone acetylation is a hallmark of the transcriptionally active parts of chromatin (Grunstein 1997). Several experiments demonstrated that the time course of histone acetylation resembles closely to that of RNA synthesis, and that higher the acetylation on the histones,

the lower the inhibition was on the RNA polymerase action (Allfrey and Mirsky 1964). However, it was initially unclear whether histone acetylation was a cause or an effect of increased transcription.

As a part of the regulation of RNA synthesis, acetylation is a reversible process as the newly synthesized histones are deacetylated upon their deposition, a modification that negatively affects transcription. Acetylation of histones is carried out by histone acetyltransferases (HATs), and their action is reversed by histone deacetylases (HDACs). Identification of these enzymes shed further light on the mechanistic regulation and functions of the histones (Bannister and Kouzarides 2011).

a. HATs

HATs are grouped into two classes depending on their cellular origins and functions. B-type HATs (HAT-Bs) are cytoplasmic that are responsible for catalyzing acetylation during the transport of newly synthesized histones from cytoplasm to the nucleus for deposition onto the newly replicated DNA (Wolffe 1992).

In contrast, A-type HATs are nuclear and more likely to be involved in the transcription related acetylation events. The first catalytic subunit of A-type HAT, p55, was identified through macromolecular preparations of a ciliated protozoan in gel-activity assays. This finding surprisingly revealed that this ciliate enzyme shows sequence similarities to a transcriptional regulator previously identified in yeast, Gcn5. This discovery provided strong molecular evidence that histone acetylation and transcriptional regulation are closely linked (Roth et al. 2001). Further studies led to the identification of other co-activators or transcriptional adaptors with intrinsic HAT activity, including mammalian GCN5 and its ortholog, PCAF, CREB-binding protein (CBP), p300, and TAFII250 (Kuo and Allis 1998).

Therefore, given the importance of histone acetylation in gene activation, it is no surprise that HAT activity would be strictly regulated as gene expression programs are rapidly changing (Brownell and Allis 1996).

b. HDACs

Histone deacetylases (HDACs) inhibit transcription in part by removing acetyl groups from lysine residues at the histone tails (Wolffe 1996; Marks et al. 2001). Over the years, the use of HDAC inhibitors from the simplest chemical, butyric acid, to more complicated inhibitors of bacterial or fungal origin,

tropoxin B or Trichostatin A (TSA) respectively, have been proven to be instrumental in studying histone acetylation. These studies made it clearer that HDACs not only act on histones but they have a variety of cellular substrates. Especially, as it will be discussed further in detail, Class IIa HDACs posses a weak deacetylase activity despite of their highly conserved deacetylase domain, suggesting the importance of other cellular substrates for their actions (Hassig et al. 1997; Marks et al. 2001).

HDACs, in general, lack DNA-binding ability and are dependent on their interaction with transcriptional activators or repressors as well as multi-protein transcriptional complexes for their recruitment to target genes. HDACs are highly conserved enzymes dating back to prokaryotes. The first HDAC, HDAC1, was identified due to its 60% similarity in the protein sequences with the yeast transcriptional repressor Rpd3p, suggesting a direct role in gene regulation (Vidal and Gaber 1991; Taunton et al. 1996). Since the identification of the first HDAC, further identified mammalian HDACs are divided into four families, Class I, Class IIa, Class IIb, and Class IV HDACs, which are referred as classical HDACs that are zinc-dependent deacetylases. In addition to these classical HDACs, another group of deacetylases that are dependent on nicotine adenine dinucleotide (NAD^+) for their catalytic activity and that show no sequence similarity to classical HDACs is the sirtuins that are also referred as Class III HDACs (Gray and Ekstrom 2001; Marks et al. 2001; Verdin et al. 2003; Haberland et al. 2009). The rest of this section will focus on class II HDACs.

Class II HDACs (HDAC4, 5, 6, 7, 9 and 10):

Class II HDACs are related to yeast protein HDA1, and taxonomic studies based on the homology within the catalytic domain have identified these enzymes in all eukaryotes as well as in the majority of the prokaryotic organisms. This class of HDACs is further subdivided into two groups, Class IIa HDACs (HDAC4, 5, 7, and 9) and Class IIb HDACs (HDAC6 and HDAC10) based on their sequence homology and domain organization. Class IIa HDACs unlike the class itself are only found in metazoans and especially these four members of this family are unique to vertebrates (HDAC4, 5, 7, and 9) (Verdin et al. 2003; Haberland et al. 2009).

Class IIa HDACs (HDAC4, 5, 7 and 9):

Class IIa HDACs are preferentially expressed in certain cell types. For example, HDAC4, 5 and 9 are particularly well expressed in brain, skeletal muscle and heart. HDAC4, in addition, shows robust expression in skeletal growth plates. The expression of HDAC7, on the other hand, is mostly restricted to endothelial cells and thymocytes (Zhang et al. 2002; Chang et al. 2004; Vega et al. 2004; Chang et al. 2006).

The structure of these HDACs consists a large N-terminal extension with conserved binding sites for transcription factors such as **Myocyte Enhancer Factor 2 (MEF2)** as further discussed in the next chapter. These transcription factors have been reported by several studies to be regulated by class II HDACs in various tissues. In addition to their N-terminal domain, this class of HDACs also possesses a highly conserved, catalytically inactive HDAC domain. One example of this is MEF2-interacting transcription repressor (MITR), which is a splice variant of HDAC9 that has been shown to inhibit MEF2 target genes, despite lacking the HDAC catalytic domain (Zhang et al. 2001a; Zhang et al. 2001b). Class II HDACs also consist conserved serine residues residing at their regulatory N-terminal region in addition to a nuclear localization signal (NLS) and a nuclear export signal (NES) within their structure. Phosphorylation of these conserved serine residues promote the binding of a chaperon protein, 14-3-3, allowing their shuttling between nucleus and cytoplasm. Therefore, this unique structure of class IIa HDACs that allows their regulation via phosphorylation, provides evidence for the regulation of transcription via extracellular signals (Fischle et al. 2001; Verdin et al. 2003; Haberland et al. 2009).

Class IIa HDACs have also been shown to recruit Class I HDACs through their C-terminal domain as one of the mechanisms of their repressive action. In addition, they could interact with other transcriptional repressors like heterochromatin protein (HP1), and C-terminal binding protein (CTBP) through their regulatory N-terminal domain. The use of mouse genetics has been instrumental in further understanding the function of this class of HDACs in vivo. Although each gene displays a tissue-specific expression regulating specific programs, it was also observed that they share commonalities in their function, pointing out similar mechanisms of action (Verdin et al. 2003; Haberland et al. 2009).

i. HDAC4

HDAC4 importance during skeletogenesis was revealed with the deletion of this gene in mice (Vega et al. 2004). Histological analyses of the bones of *Hdac4-/-* mice demonstrated that these mice have increased, immature ossification of their bones, which could also explain the perinatal lethality of these mice. As most of their ribs are formed from mineralized bone, this prevents the expansion of the ribs and therefore proper breathing. Another striking feature of this phenotype was the similarities between the skeletal abnormalities observed in these mice compared to those observed in mice overexpressing Runx2, a transcription factor expressed in proliferating chondrocytes promoting chondrocyte differentiation. Further analysis of the mice supported by the molecular data demonstrated that HDAC4 interacts with and inhibits Runx2 activity in chondrocytes, therefore leading to immature endochondral bone formation (Vega et al. 2004). Through the studies performed in chick chondrocytes, HDAC4 was also demonstrated to inhibit chondrocyte hypertrophy downstream of parathyroid hormone-related peptide (PTHrP) signaling by inhibiting MEF2c function in chondrocytes (Kozhemyakina et al. 2009). In addition, through its association with HDAC5, HDAC4 was also shown to regulate muscle differentiation (McKinsey et al. 2000).

ii. HDAC5 and HDAC9

With the use of mouse genetics, HDAC5 and 9 have been shown to have redundant functions in inhibiting the cardiac growth upon stress. Mice with the deletion of either *Hdac5* or *Hdac9* are viable (McKinsey et al. 2000; Zhang et al. 2002), however the compound mutant mice lacking both of these genes die due to ventricular septal defects and thin-walled myocardium which are caused by abnormalities in the growth and maturation of cardiomyocytes (Chang et al. 2004). Upon stress, adult heart responds in a pathological growth response that ultimately causes loss of cardiac function. Mice lacking HDAC5 or HDAC9 are hypersensitive to cardiac stress caused by excess workload or neuro-humoral signaling. Given the known interaction of this class of HDACs with MEF2 family transcription factors as well as the importance of function of MEF2 proteins during cardiomyocyte differentiation further studies described that the cardiac defects observed in the compound mutant mice are a result of over activation of MEF2c transcription factor. In addition to their function during cardiac hypertrophy, HDAC5

and 9 along with HDAC4 have been shown to play a large role in skeletal muscle function, another tissue where these proteins are highly expressed (Potthoff et al. 2007).

Skeletal muscles consist two different forms of fibers that have different contractility and metabolic functions. Slow-twitch or type1 myofibers are the highly vascularized fibers rich in mitochondria, exhibiting an oxidative metabolism and are resistant to fatigue, whereas fast-twitch or type II myofibers are involved in rapid bursts of contraction using a glycolytic metabolism and could fatigue rapidly. MEF2 transcription factors are regulators of slow-twitch myofiber phenotype and this function of this family of transcription factors is inhibited by Class IIa HDACs. Deletion of either one of these Class IIa HDACs in skeletal muscle results in the conversion of type II fibers to type I fibers as the inhibition on MEF2 is released (Potthoff et al. 2007).

In addition to these 2 functions, HDAC9 is also important in modulating the response of skeletal muscle to motor intervention. Electrical activity from the neurons represses the muscle specific gene expression such as those encoding the acetylcholine receptor subunits. Therefore, upon denervation, as the gene expression is repressed, muscle fibers become hypersensitive to acetylcholine. Deletion of HDAC9 in mice results in extreme sensitivity to changes in gene expression upon denervation, and overexpression of this gene results in an opposite phenotype where the skeletal muscles are rendered insensitive to denervation (Haberland et al. 2007).

iii. HDAC7

HDAC7 is specifically expressed in the endothelial cells in the inner lining of cardiovascular system. The deletion of *Hdac7* in mice results in embryonic lethality due to loss of integrity between endothelial cells that eventually causes rapture of the blood vessels leading to hemorrhaging. This phenotype of these mice is accompanied with increased expression of matrix metalloproteinase 10 (MMP10) that degrades the extracellular matrix perturbing the interactions of the endothelial cells with the smooth muscle cells. It was further shown that in mice lacking *Hdac7*, MEF2 activity is elevated to pathological levels. This function of HDAC7 in the regulation of MEF2 to maintain vascular integrity has important implications in various human disorders such as in vascular leakages observed in atherosclerosis and aneurysms (Chang et al. 2006).

Class IIb HDACs (HDAC6 and HDAC10):

This subclass of class II HDACs is characterized by the presence of two HDAC catalytic domains, which is only a partial duplication in HDAC10. Similar to class IIa HDACs, this class of HDACs also shows some degree of specificity in their expression, such as HDAC6 is predominantly expressed in testes whereas expression of HDAC10 is seen in liver, spleen and kidney (Fischle et al. 2001). The analysis of the catalytic activity of two HDAC domains in HDAC6 with site-directed mutagenesis showed that these two domains might function independently from each other, however the separation of these domains from each other results in the loss of enzymatic activity (Grozinger et al. 1999).

The use of HDAC inhibitors such as sodium butyrate or trapoxin showed that the enzymatic activity of HDAC6 and HDAC10 is more resistant to these inhibitors compared to that of both class I and class IIa HDACs. The sensitivity to both sodium butyrate and trapoxin is restored in HDAC10 when the second incomplete HDAC domain is removed suggesting that these two domains in HDAC10 might functionally interact (Furumai et al. 2001).

Despite the use of a pharmacological nuclear export inhibitor on both HDAC6 and HDAC10, the cytoplasmic localization of these HDACs is not changed as it was shown for HDAC6 that its subcellular localization is dependent on a strong nuclear export signal (NES1) located within its N-terminal. For HDAC10, several putative export sequences have been reported however their true functionality is still under further investigation.

Furthermore, HDAC6 was shown to act as a specific tubulin deacetylase mainly being localized with the microtubule network in the cytoplasm. It was also demonstrated that overexpression of HDAC6 results in the chemotactic movement of the cells which is a process dependent on the microtubules (Zhang et al. 2008).

2. MEF2 Family Of MADS Box Family Transcription Factors

MADS box is a conserved motif of 56 amino acids found within the DNA binding domain of various eukaryotic transcription factors. In general, MADS box is within the N-terminal of the protein. The four transcription factors that contain this conserved domain, MCM1 (Mini Chromosome Maintenance 1), AG (Agamous), DEFA (Deficiens) and SRF (Serum Response Factor), are the founder members of this family of transcription factors where the name itself is also derived from (Schwarz-Sommer et al. 1990).

Myocyte Enhancer Factor 2 (MEF2) proteins, members of MADS box family of transcription factors, were initially cloned based on their sequence homology to SRF. This group of proteins was also referred as RSRFCs (Related to Serum Response Factor). There is only one MEF2 gene in organisms like *Saccharomyces cerevisiae, Drosophila* and *Caenorhabditis elegans* whereas vertebrates have four MEF2 proteins identified, MEF2a, MEF2b, MEF2c, and MEF2d. MEF2 proteins were identified due to their binding through a conserved A+T rich region found in the promoters of muscle creatinine kinase (MCK) enhancer that was essential for full activity of the enhancer. Furthermore, MEF2 binding sites were found in the promoter regions of many muscle specific genes as they are the transcriptional activators for these genes (Shore and Sharrocks 1995). MEF2 DNA binding consensus sequence, $YTA(A/T)_4TAR$, is a distinct sequence that is only unique to MEF2 family transcription factors. MEF2A, C and D have different binding affinities to the same consensus sequence (Potthoff and Olson 2007).

MEF2 proteins share more than 80% amino acid homology within their MADS-box domain that is located within the amino terminal. Adjacent to this domain, they also contain a MEF2 domain, which is unique to this group of transcription factors. These two domains promote dimerization, DNA binding and co-factor interactions of these proteins. Although MEF2 proteins are transcriptional activators, for their functions they still rely on the recruitment of and cooperation with other transcription factors or co-activators. The C-terminal of this family of transcription factors is the transcriptional activation domain, which is subjected to numerous alternative splicing, making it quite divergent between the members of this family (Potthoff and Olson 2007).

MEF2 proteins have distinct as well as overlapping expression patterns. In vertebrates the highest expression of MEF2 proteins is observed in muscle and brain, however these proteins are also expressed in endothelium, lymphocytes, neural crest, smooth muscle and bone. Expression of MEF2

proteins in many cell types such as chondrocytes, neurons and muscle occur concurrently with the activation of several differentiation programs and it is the balance between the activating functions of the MEF2 proteins along with the repressive functions of Class IIa HDACs on these transcription factors that regulate the development of these tissues (Potthoff and Olson 2007) (FigureI.1).

3. Bone (Re)modelling

Skeleton is a dynamic organ composed of two distinct tissues, cartilage and bone, and three specific cell types; chondrocytes in cartilage; osteoblasts and osteoclasts, which are the two major cell types in bone. Among these cells, chondrocytes and osteoblasts share the same mesenchymal origin; being derived from osteo-chondro progenitors and are regulated by an elaborate transcriptional machinery to control and distinguish their differentiation, whereas, osteoclasts belong to monocyte/ macrophage lineage (Karsenty et al. 2009).

In the last 20 to 30 years, many advances have been made in understanding the molecular bases of skeletal development and functions. In addition to a large cohort of transcription factors, mouse genetics has placed the skeleton in the center of diverse physiological network. This latter aspect is the focus of this thesis.

In addition to expanding at a very slow rate during adulthood, bone is a dynamic organ that is continually remodeled. Bone (re)modeling is a lifetime process, during which mineralized bone is removed by osteoclasts, the bone resorbing cells, followed by formation of mineralized bone matrix by osteoblasts that are the bone forming cells.

i. Osteoblasts and bone formation

Osteoblasts, cells of mesenchymal origin, are multifunctional cells. They are responsible for synthesis of proteins of the bone matrix, osteoclast differentiation, and last but not least, they are the endocrine cells of the bone secreting two hormones, osteocalcin and FGF23 (Karsenty 1999; Fukumoto and Martin 2009; Karsenty and Ferron 2012).

Among the transcriptional machinery regulating osteoblast differentiation and function, *Runx2*, in addition to being a regulator of chondrocyte hypertrophy, is also the master gene of osteoblast differentiation (Ducy et al. 1997). Bone matrix consists mainly of type I collagen, which forms the 90% of its protein content. Runx2 also regulates the amount of bone matrix to be deposited by already differentiated osteoblasts, by promoting the expression of *αI(I) collagen* that consists a Runx2 binding site within its promoter (Ducy et al. 1997). Consistent with its function, mice lacking *Runx2* have no osteoblasts, even though they have a normally patterned skeleton that is exclusively formed of cartilage

(Komori et al. 1997; Otto et al. 1997). Moreover, the mice with haploinsufficiency in the *Runx2* locus, display delayed ossification of certain bones of the skull as well as hypoplastic clavicles (Otto et al. 1997). These abnormalities are similar to what is observed in a human skeletal dysplasia known as cleidocranial dysplasia (CCD). The CCD patients are heterozygous for loss-of-function mutations in the *Runx2* gene (Lee et al. 1997; Zhou et al. 1999).

The differentiation of osteoblasts is also marked by the activation of an array of genes like *alkaline phosphatase (ALP), type I collagen, bone sialoprotein (BSP)* and *Osteocalcin*. Osteocalcin is the most abundant non-collagenous protein of bone and it is expressed only in differentiated osteoblasts marking the mature osteoblasts (Hauschka et al. 1989; Ducy et al. 1997; Karsenty 2008).

Another transcription factor regulating bone formation by promoting osteoblast differentiation is ATF4 whose function is also implicated in a human disease, Coffin-Lowry syndrome (Yang et al. 2004). Deletion of *Atf4* in mice results in delayed skeletal development and a severe decrease in bone mass due to a decrease in bone formation (Yang et al. 2004). At the molecular level, ATF4 binds to OSE1 site on the *Osteocalcin* promoter, thereby inducing *Osteocalcin* expression (Ducy and Karsenty 1995; Schinke and Karsenty 1999; Yang et al. 2004). ATF4 is also required for osteoblast function and bone formation in adult mice as it is necessary for the synthesis of type I collagen, a hallmark of the bone matrix, through its role in regulation of the amino acid transport (Yang et al. 2004).

In addition to its function in bone formation and promoting *Osteocalcin* expression, ATF4 is also important for another function of osteoblasts. ATF4 induces the expression of *Rankl* (Receptor for activation of Nuclear Factor kappa B (NF-κB) (RANK) ligand), a molecule secreted by osteoblasts to promote osteoclast differentiation and thereby, bone resorption.

ii. Osteoblasts and bone resorption

In vitro experiments performed in 1990 demonstrated that maturation of macrophages to osteoclasts required the presence of marrow stromal cells or cells of the osteoblast progeny (Udagawa et al. 1990). This was due to osteoblasts secreting in addition to *Rankl,* another molecule, *M-csf* (Macrophage colony-stimulating factor) that are both necessary and sufficient for differentiation of osteoclasts (Udagawa et al. 1990).

Osteoclasts are the cells responsible for bone resorption. They are multinucleated cells formed by the fusion of mononuclear monocytes (Teitelbaum 2000). The initial experiments on mice with osteopetrosis, a disease characterized by an increase in bone mass due to a failure in the function of osteoclasts, demonstrated that injection of normal spleen cells in these mice corrected their bone phenotype, suggesting a hematopoietic origin for osteoclasts (Walker 1973). Later on, the hematopoietic origin of osteoclasts was also established in humans (Coccia et al. 1980).

What prompted the discovery of RANKL as an osteoclast differentiation factor is another molecule that is also secreted by osteoblasts, osteoprotegerin (OPG). OPG is the soluble decoy receptor for *Rankl* that competes with RANK receptor on osteoclast progenitors (Simonet et al. 1997). Overexpression of *Opg* results in osteopetrosis due to a decrease in bone resorption whereas the mouse models with deletion of *Opg* display a massive decrease in their bone mass due to an increase in bone resorption displaying severe osteoporosis (Simonet et al. 1997; Bucay et al. 1998; Mizuno et al. 1998).

Bone resorption is a highly regulated multistep process, which includes the proliferation of immature osteoclast precursors, differentiation of these cells to osteoclasts and lastly the degradation of mineralized bone matrix by mature osteoclasts (FigureI. 2). Initial step of bone resorption is the attachment of osteoclasts to the target matrix. Bone is formed of type I collagen and non-collagenous proteins containing a mineral phase of substituted hydroxylapatite and initially the inorganic phase of bone desolubilizes which is followed by the degradation of the matrix (Blair et al. 1986). Osteoclasts achieve the demineralization of the bone by forming an acidified microenvironment called a resorption lacuna that is mediated by a vacuolar H^+-adenosine triphosphatase (ATPase) (Blair et al. 1989). The pH of the resorption lacuna is maintained by an abundant proton transport via an energy independent Cl^- /HCO_3^- exchanger on the cell's antiresorptive surface (Teti et al. 1989). Therefore the ion transporting in the resorptive microenvironment promotes a pH of approximately 4.5 (Silver et al. 1988). This acidic environment mobilizes the bone mineral, followed by the demineralized organic matter of bone being degraded by a lysosomal protease, cathepsin K and later being endocytosed by osteoclasts (Nesbitt and Horton 1997) (FigureI. 3).

Given that bone remodeling is a continuous process necessary for maintenance of bone mass, osteoblasts maintain the balance between bone resorption and bone formation by balancing the

expression of *Rankl* and *Opg* that determines the amount of bone to be resorbed (Hofbauer 1999). Thus hormones and factors, which regulate *Rankl* expression in osteoblasts become of great importance in understanding the regulation of bone resorption (FigureI. 3). Such examples of major extracellular signals that are known to induce *Rankl* expression in osteoblasts are the parathyroid hormone (PTH) signaling and the sympathetic signaling, which will be further discussed in the next sections (FigureI. 3).

iii. Endocrine functions of osteoblasts

Aside from being the bone forming cells and regulating osteoclastogenesis through their secretion of *Rankl* and *Opg*, osteoblasts are also endocrine cells secreting at least, two hormones, FGF23 and osteocalcin. Among these, osteocalcin is a member of a larger family of proteins called GLA proteins. It contains 3 glutamic acid residues (GLA residues) that are γ-carboxylated by a specific vitamin K-dependent γ-carboxylase.

Osteocalcin displays several features of a hormone. First, osteocalcin is initially synthesized as a pre-promolecule and secreted in the general circulation. Later through post-translational modifications, the glutamic acid residues of osteocalcin undergoes carboxylation to form the γ -carboxyglutamic acid (Gla) residues, a form of osteocalcin that has higher affinity for hydroxyapatite (HA) than the undercarboxylated osteocalcin (Hauschka et al. 1989). The last evidence that suggested that *Osteocalcin* is more than a bone- specific gene, hinting that skeleton may regulate energy metabolism via this gene was the observation of increased visceral fat, glucose intolerance and hypoinsulinemia that are displayed by *Osteocalcin -/-* mice (Lee et al. 2007).

a. Regulation of energy metabolism by bone

Further studies on the function of bone in regulation of energy metabolism demonstrated that the undercarboxylated form of osteocalcin is the biologically active form (Lee et al. 2007; Ferron et al. 2010). Osteocalcin regulates energy metabolism when undercarboxylated, specifically on the 13^{th} carbon of the glutamic acid residue (GLU13) that is stimulated upon increase in bone resorption. The circulating levels of undercarboxylated osteocalcin favors proliferation, *Insulin* expression and insulin secretion in β-cells, as well as insulin sensitivity in the peripheral tissues (Ferron et al. 2010) (FigureI. 4-5).

b. Regulation of male fertility by bone

One of the most powerful hormonal regulation of bone remodeling is via the sex steroid hormones, which is most commonly exemplified with the loss of gonadal function in post-menopausal women causing osteoporosis due to increased bone resorption (Pacifici 1998). Considering that gonads regulate bone remodeling, this brought up the question of whether bone could talk back to gonads to regulate them.

First through cell culture experiments, it was shown that Leydig cells of the testes when cultured with the supernatant of osteoblasts, enhanced testosterone production, however this supernatant had no effect on the estrogen synthesis by the cultured ovaries. This experiment suggested that a secreted molecule must be responsible for affecting the androgen synthesis in the testes. Since, osteocalcin has been identified as an osteoblast-specific hormone regulating whole body glucose homeostasis and energy expenditure (Lee et al. 2007), and based on the hypothesis that energy metabolism, bone mass and reproduction are connected, along with the poor breeding observed in *Osteocalcin-/-* mice raised the hypothesis that osteocalcin might be signaling from bone to the testes. The mice deficient of *Osteocalcin* had a significant decrease in their testes size and weight, in addition to a decrease in their seminal vesicle and epididymes weights and sperm count, whereas in mice lacking *Esp* (Embryonic stem cell phosphatase), a negative regulator of osteocalcin bioactivity, these parameters were increased. It was further demonstrated that *Osteocalcin* exerts its action on the Leydig cells of testes through its receptor, OSTR or Gpcr6a, by activating a cAMP -dependent pathway to promote activation of the transcription factor, cAMP-binding response element (CREB) therefore up-regulating the genes necessary for testosterone biosynthesis (Oury et al. 2011). Specific deletion of *Gpcr6a* in Leydig cells of the testes, not only replicated the phenotype observed in mice lacking this gene globally but also it was a reflection of what was observed in *Osteocalcin -/-* mice (Pi et al. 2008; Oury et al. 2011).

It was further established that insulin signaling in osteoblasts also favors male fertility by promoting the activation of osteocalcin through increased bone resorption (Ferron et al. 2010; Oury et al. 2013a). Studies performed on patients with peripheral testicular insufficiency with unknown cause revealed that couple of these patients are heterozygous for a point mutation in the transmembrane region

of *Gpcr6a* resulting in a deleterious substitution, confirming the function of osteocalcin in male fertility in humans as well (Oury et al. 2013a) (FigureI. 5).

c. Regulation of cognition and memory by bone

Lastly, osteocalcin favors brain development and cognitive functions. The existence of strong regulation of bone mass exerted by brain raised the question of whether in turn, bone as an endocrine organ through osteocalcin regulates this or other functions of the brain (Takeda et al. 2002; Yadav et al. 2009; Oury et al. 2010). One striking observation about the mice that lack *Osteocalcin* set the bases for this work. Over the years, investigators working with *Osteocalcin-/-* mice have realized their docile nature compared to WT mice. When analyzed in dark/light cycles, it was also confirmed that these mice have decreased locomotion. Furthermore, it was observed that these mice are more anxious and depressed compared to WT mice, and have defects in memory and learning which extends to the maternal effect of osteocalcin. By subcutaneous infusion of mice with an osteocalcin pump demonstrated that osteocalcin crosses the blood-brain barrier. In addition, osteocalcin is also a hormone that crosses the placenta as demonstrated by the offspring of *Osteocalcin-/-* mice that display severe defects in spatial learning and memory. Maternal osteocalcin was shown to promote fetal neurogenesis in mice that do not have this hormone. However, the action of osteocalcin in the brain is not through its only known receptor, OSTR, since mice lacking this receptor do not display any of the cognition or memory defects observed in *Osteocalcin-/-* mice. Therefore, the receptor through which osteocalcin exerts its action on brain still needs to be identified (FigureI. 5).

4. Systemic Regulation Of Bone Resorption

The variety of the functions of osteoblasts leaves no doubt on the strict regulation of gene expression in these cells both intrinsically and via extracellular signals. This section will discuss in further detail the two extracellular cues that affect osteoblast function of bone resorption through different transcriptional means that are mediated by the same second messenger.

i. Parathyroid hormone signaling

Parathyroid hormone (PTH) is a major endocrine regulator of mineral homeostasis and bone mass. The initial appearance of PTH during evolution starts with the appearance of terrestrial animals and their movement from an aquatic environment to a Ca^{2+} deficient environment. The primary function of PTH is to control the Ca^{2+} homeostasis in the body considering that Ca^{2+} is an important mineral necessary for healthy neuromuscular function, bone mineralization and many other physiological processes (Marcus et al. 2001). PTH secretion is stimulated from the parathyroid glands upon decrease in the plasma Ca^{2+} levels. PTH acts directly on bone and kidney and indirectly on intestine to restore the Ca^{2+} levels in the blood (Brown 1991; Potts et al. 1997; Silverberg et al. 1999). Further studies established PTH as one of the major mediators of bone remodeling. Initially, the PTH action on bone was observed to promote bone resorption within 15 minutes of its administration (Holtrop and King 1977).

Human PTH is 1-84 amino acid long peptide, which further gets processed into a shorter peptide of 1-34 amino acids, acting directly on kidneys and osteoblasts in bone, through PTH1 receptor, PTH1R (Silve et al. 1982; Teitelbaum et al. 1982; Goltzman et al. 1986)**.** 1-34 amino acid sequence of PTH is known to bind and be sufficient for activating PTH1R (Mannstadt et al. 1999) (FigureI. 6).

Several studies have shown that PTH1R is expressed only the osteoblastic cells in bone and that PTH alone was not able to promote differentiation of isolated osteoclasts in vitro unless osteoblasts-like cells were present in the environment (Goltzman et al. 1986; Jilka 1986). It was further established that direct contact between the osteoclasts and osteoblast-like cells were required for PTH-induced activation of osteoclasts. The explanation for these findings came from the discovery of RANKL and RANK receptor and their function in the regulation of osteoclastogenesis (Teitelbaum 2000). It is now established that PTH induces bone resorption indirectly by acting on osteoblasts to promote *Rankl* expression and

secretion, which then allows osteoclast differentiation and maturation, therefore increased bone resorption (O'Brien 2010). However, it is not clearly elucidated yet what are the molecular events initiated by PTH to enhance *Rankl* expression.

PTH exerts its functions through PTH1R to mediate the regulation of PTH-dependent mineral homeostasis or paracrine actions of PTHrP during chondrogenesis (Abou-Samra et al. 1989; Mannstadt et al. 1999). PTH1R is a G-protein coupled receptor with seven membrane-spanning helices, which further includes a large amino-terminal extracellular domain and an intracellular carboxyl-terminal domain similar to the family B (Class II) receptors that it belongs to. PTH1R can activate at least two second messenger dependent signal transduction pathways, **cyclic–AMP (cAMP) dependent/protein kinase A (cAMP/PKA)** or the phospholipase C/protein kinase C (PLC/PKC) pathways to mediate the functions of PTH and PTHrP to promote gene expression. cAMP is a second messenger involved in the action of various hormones and extracellular regulatory signals. PTH through its binding to PTH1R activates an enzyme called adenylyl cyclase (AC) to increase the cAMP levels in the cell. In turn, increasing levels of cAMP activates cAMP-dependent protein kinase A, PKA, which phosphorylates and therefore regulates proteins that are involved in physiological responses (FigureI. 7). PTH1R also activates PLC that is an enzyme responsible for producing diacylglycerol (DG) and soluble 1,4,5-inositol triphosphate (IP_3), which also act as second messengers. DG and IP_3 activate protein kinase C (PKC) and through their direct action on calcium channels increasing cytosolic-free calcium levels in the cell to transduce signals (FigureI. 7).

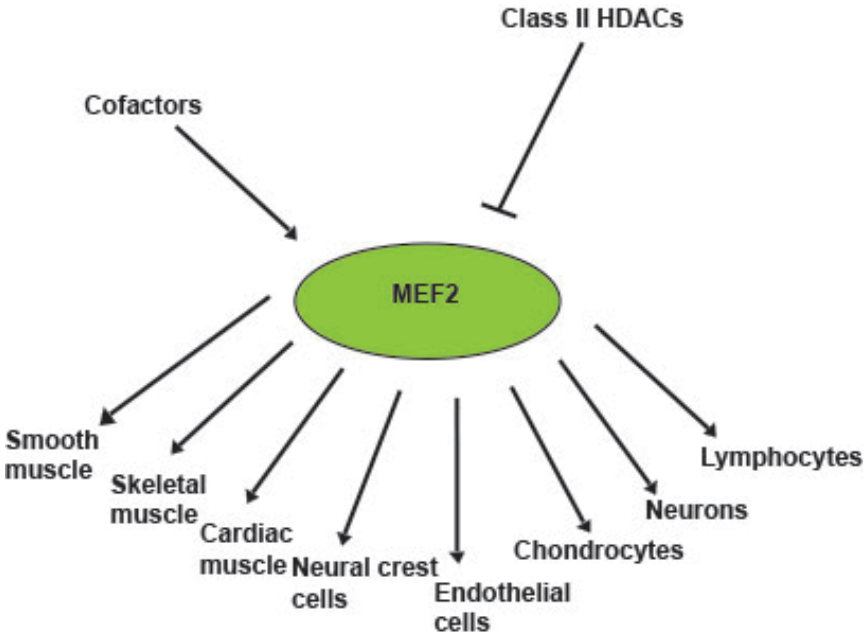
Numerous studies have demonstrated that PTH1R is required for PTH stimulated bone resorption and that the action of PTH mediated by this receptor is through the activation of cAMP/PKA dependent pathway (Avioli and Krane 1990; Kondo et al. 2002). Cell-based assays also demonstrated that cAMP/PKA-dependent signaling of PTH requires cAMP-binding response element (CREB) action (Fu et al. 2002). However, in vivo deletion of *Creb* specifically in osteoblasts shows no abnormalities in *Rankl* expression or bone resorption suggesting that transcriptional means of PTH action is yet to be identified (Kajimura et al. 2011).

ii. Sympathetic signaling

The sympathetic nervous system is another strong regulator of *Rankl* expression in osteoblasts (Elefteriou et al. 2005). Sympathetic signaling acts through its receptors β-adrenergic receptors (Adrβ). These receptors belong to a family of GPCRs that act by increasing intracellular **cAMP levels** and therefore activating PKA to promote phosphorylation of downstream targets controlling physiological events. β-adrenergic receptors are divided into sub-groups depending on their affinities to different agonists and antagonists: β1, β2 and β3 receptors (Takeda et al. 2002).

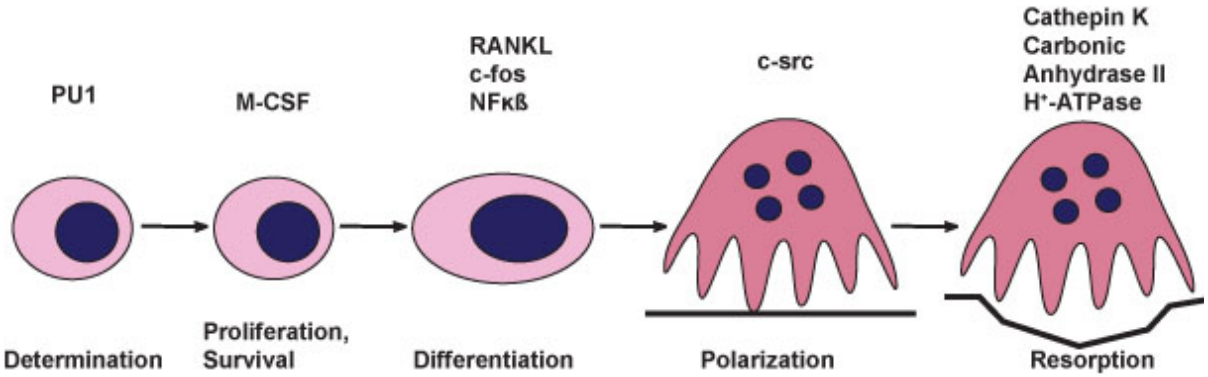
The *Adrenergic β_2 receptor ($Adr\beta_2$)* is the only adrenergic receptor expressed in osteoblasts. The importance of sympathetic signaling in regulation of bone remodeling was demonstrated through deletion of *$Adr\beta_2$ receptor*. Mice deficient of this receptor display a high bone mass phenotype (Takeda et al. 2002). Besides an increase in bone formation parameters, *$Adr\beta_2$-/-* mice also display a significant decrease in their osteoclasts surface and deoxypyridinoline (Dpd) levels, a marker of osteoclast function. Further molecular analysis led to the understanding that sympathetic signaling promotes bone resorption through osteoblasts by inducing *Rankl* expression in a cAMP-dependent manner (Elefteriou et al. 2005). This action of sympathetic signaling in osteoblasts occurs through the transcription factor ATF4, which binds to the *Rankl* promoter inducing *Rankl* expression and thereby promoting bone resorption (Elefteriou et al. 2005).

Aside from its function in regulating the bone resorption arm of bone remodeling, sympathetic signaling was also shown to indirectly regulate endocrine function of osteoblasts by affecting the bioactivity of *Osteocalcin* through *Esp*. The regulation of insulin secretion in β-cells of the pancreas in opposite ways via leptin and osteocalcin led to the discovery of the function of sympathetic signaling in the regulation of the energy metabolism (Hinoi et al. 2008). The hypoglycemic, hyperinsulinemic phenotype of *$Adr\beta_{2osb}$-/-* mice was due to the down- regulation of *Esp* expression. It was further demonstrated that sympathetic signaling promotes *Esp* expression also through the transcription factor ATF4 (Hinoi et al. 2008; Yoshizawa et al. 2009).



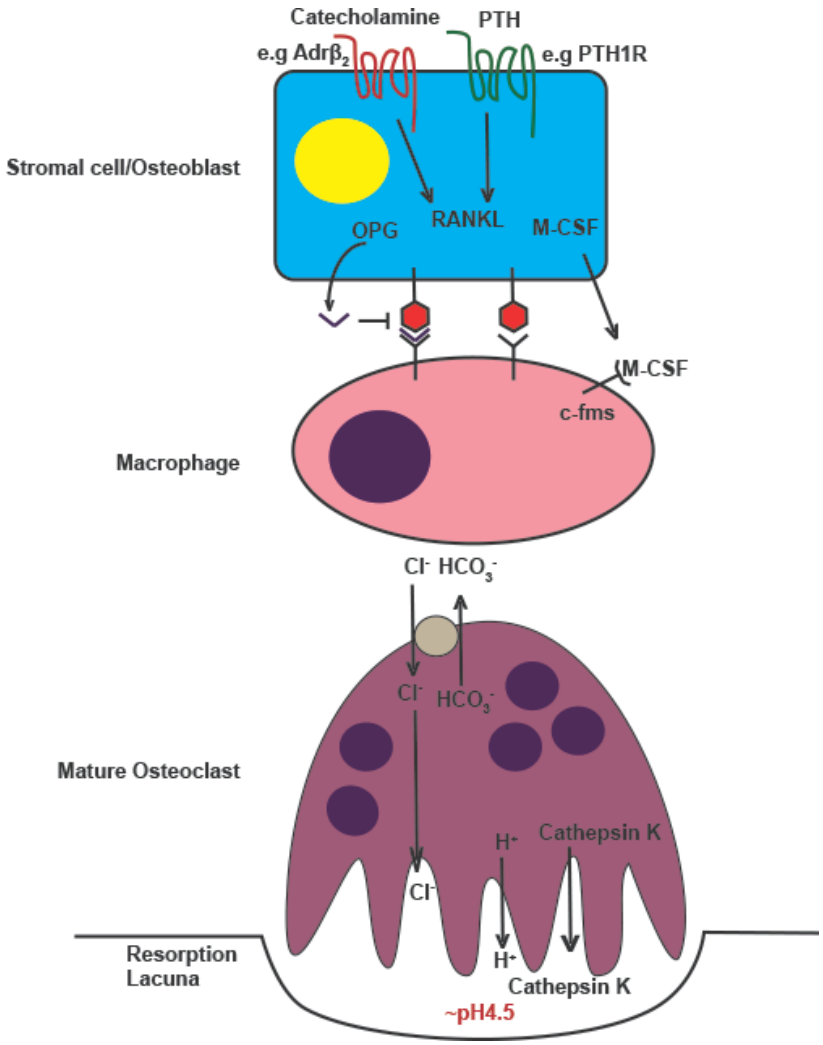
Figurel. 1. MEF2 proteins as central regulators of differentiation

The tissues where the function of MEF2 proteins have been shown to promote many cellular processes such as differentiation. Modified from (Potthoff and Olson 2007).



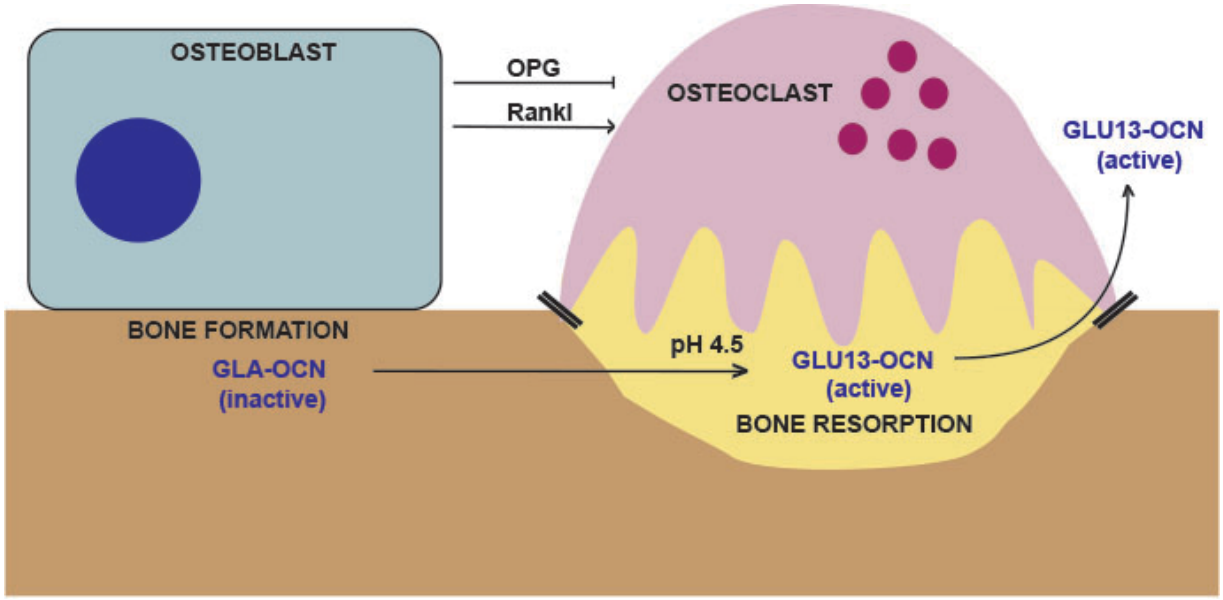
Figurel. 2. Osteoclastogenesis

The studies on osteoporotic mouse models identified genes that play a major role in different stages of osteoclastogenesis. *PU1* is essential for differentiation into the early macrophage precursors. Absence of *M-csf* results in the formation of immature macrophages. Later during differentiation step, *Rankl* being the most important, *c-fos* and *NFκB* are necessary for normal macrophage differentiation into mature osteoclasts. *c-src* has also been shown to be important for polarization of the plasma membrane of osteoclasts and the absence of carbonic anhydrase II and H^+-ATPase was shown to affect the resorbing ability of osteoclasts as the acidification of the resorptive environment is inhibited. Last but not least, cathepsin K is needed for degrading the organic matrix of bone Modified from (Teitelbaum 2000).



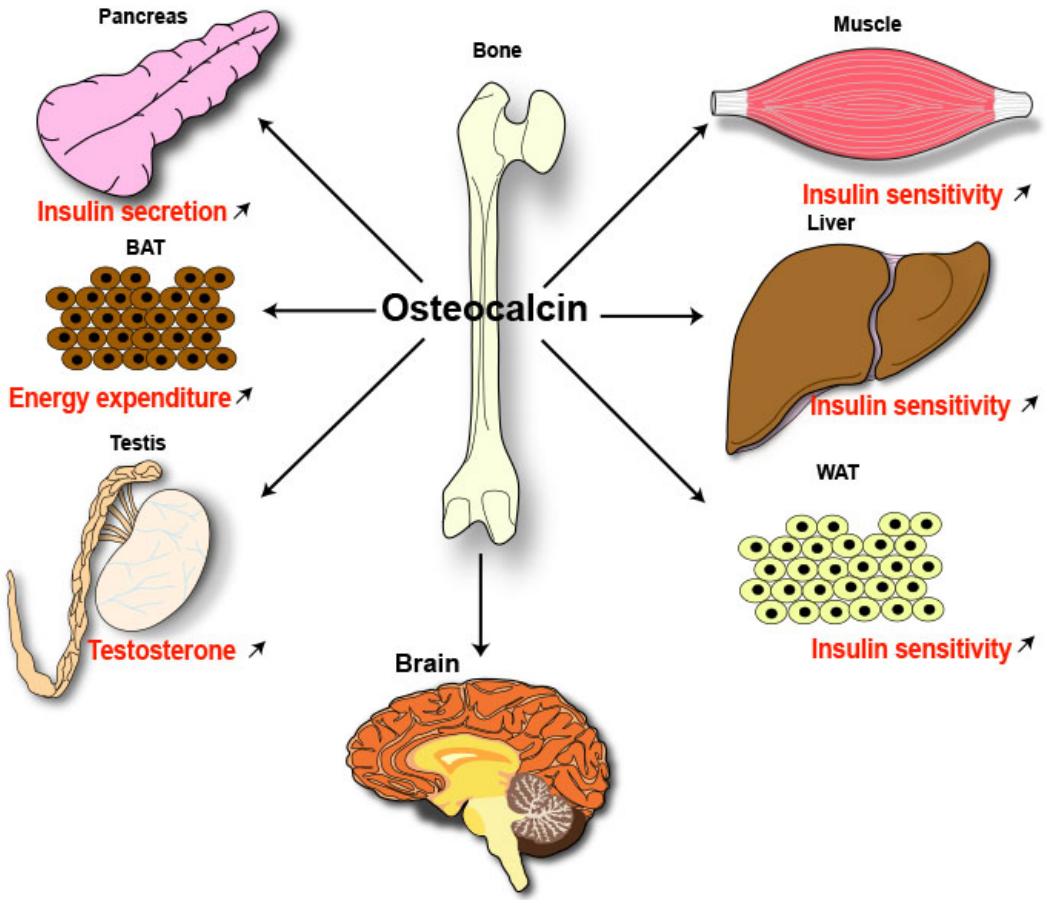
Figurel. 3. Mechanisms of Osteoclastogenesis

RANKL and M-CSF are synthesized by the osteoblast to promote the differentiation of macrophage progenitors into mature osteoclasts. RANKL binds to its receptor, RANK receptor, and M-CSF to c-fms. The expression of *Rankl* is upregulated by extracellular signals, e.g. PTH or catecholamines. The process of osteoclastogenesis is also negatively regulated by osteoblasts through the secretion of another molecule, OPG. Mature osteoclasts resorbs the bone through the use of Cl^-/HCO_3^- channels to maintain an acidic pH of 4.5, as well as by their secretion of molecules e.g. Cathepsin K, a lysosomal protease. Modified from (Teitelbaum 2000).



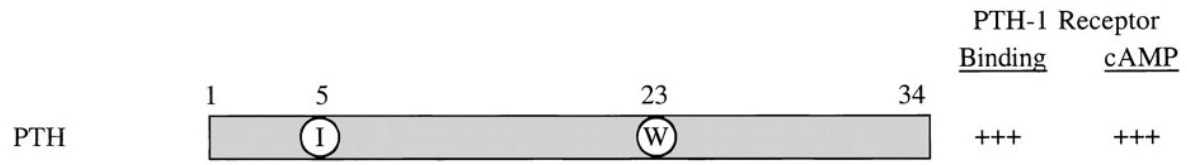
Figurel. 4. Regulation of osteocalcin bioactivity via bone resorption

RANKL promotes osteoclast differentiation and bone resorption whereas OPG inhibits this process. As the osteoclasts resorb bone, they form an acidic resorption lacuna of pH 4.5 that promotes decarboxylation and thereby, activation of osteocalcin. Circulating active osteocalcin promotes insulin secretion and sensitivity, as well as male fertility, and memory and cognition. Modified from (Karsenty and Ferron 2012).



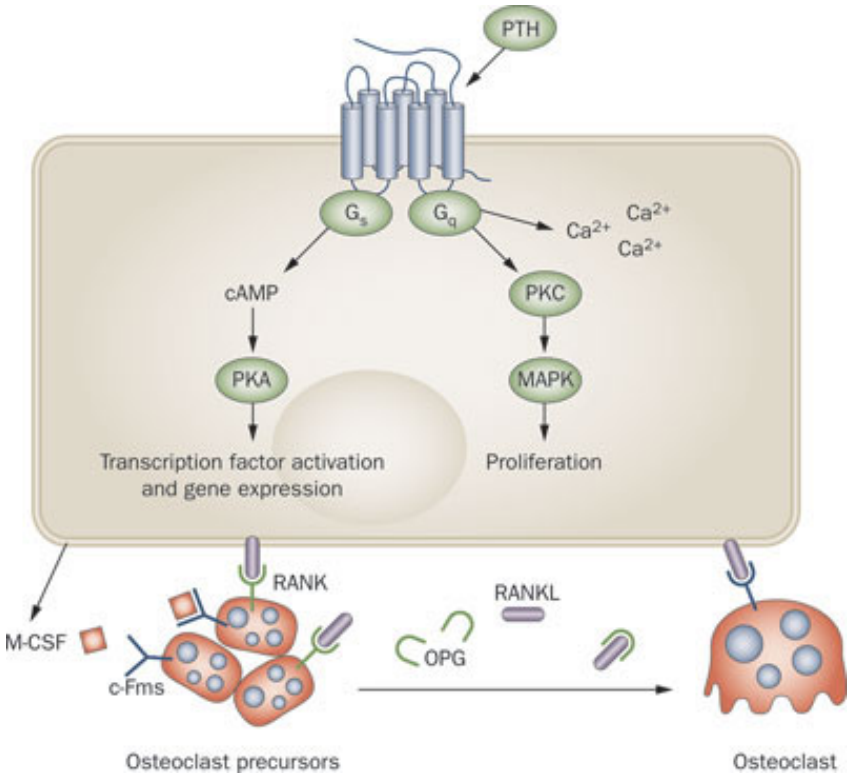
Figurel. 5. Spectrum of the functions of bone-derived hormone osteocalcin

Circulating active osteocalcin in the body promotes insulin sensitivity in insulin sensing tissues such as muscle, white adipose tissue (WAT), and liver; increases insulin secretion and β-cell proliferation in pancreas. Increases energy expenditure through brown adipose tissue (BAT); promotes testosterone production therefore favoring male fertility, and last but not least favors memory and cognition through its direct action on brain (Modified from (Karsenty 2011)).



Figurel. 6. The schematic representation of PTH

1-34 amino acid of PTH has the ability to bind and promote cAMP production through PTH1R (Mannstadt et al. 1999)



Figurel. 7. Model of PTH action on osteoblasts through PTH1R

PTH binds to PTH1R, a GPCR, on osteoblasts to activate either Gs protein that promotes a cAMP-mediated transcriptional control of osteoclastogenesis or Gq protein to activate a Ca^{2+}/PKC pathway that has been shown to promote osteoblast proliferation (Karsenty 2011).

CHAPTER II. MANUSCRIPT

Preface

The completion of this work is owing to the contribution from many people. Dr. Arnaud Obri has been instrumental in the development of the project. He has confirmed several results I have obtained and performed most of the molecular work. *Hdac5-/-, Mef2af/f* and *Mef2cf/f* mice were originally generated and provided by Dr. Eric Olson. *Ppr1f/f* mice were generated and provided by Dr. Henry Kronenberg and *Smurf2f/f* mice are generated and provided by Dr. Hong Zhang. Dr. Franck Oury helped me in performing the dissections for the testes parameters as well as in the establishment of Morris Water Maze test. Dr. Jianwen Wei and I performed the GSIS test on $Hdac4_{osb}$-/- mice. Under the instruction Dr. Gerard Karsenty, I performed the rest of the described work.

HDAC4 integrates PTH and sympathetic signaling to regulate multiple functions of osteoblasts

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Abstract

Both parathyroid hormone (PTH) and the sympathetic tone promote *Rankl* expression in osteoblasts and osteoclast differentiation by enhancing cAMP production, through an unidentified transcription factor for PTH and ATF4 for the sympathetic tone. How two extracellular cues using the same second messenger in the same cell elicit different transcriptional events is unknown. Here we show that PTH favors *Rankl* expression by triggering the ubiquitination of HDAC4, a class II histone deacetylase, partly via Smurf2. HDAC4 degradation releases MEF2c that transactivates the *Rankl* promoter. On the other hand, sympathetic signaling in osteoblasts favors the accumulation of HDAC4 and its association with ATF4. In this setting, HDAC4 increases *Rankl* expression. Through this interaction with ATF4, HDAC4 also influences *Osteocalcin* expression, and its endocrine and cognitive functions. This study shows that through its ability to differently connect distinct extracellular cues to their genome, HDAC4 is a global regulator of osteoblast functions.

Introduction

Osteoblasts are bone-specific, multifunctional cells. Through their synthesis of proteins of the bone extracellular matrix, they are responsible for bone formation (Karsenty et al. 2009). Because osteoblasts express the osteoclast differentiation factor, RANKL, they also favor bone resorption (Simonet et al. 1997; Teitelbaum 2000). In addition, osteoblasts secrete two hormones, FGF23 and osteocalcin (Fukumoto and Martin 2009). The diversity of their functions explains why osteoblasts are influenced by multiple extracellular cues.

One of them, the parathyroid hormone (PTH) regulates *Rankl* expression through several mechanisms (Tawfeek et al. 2010). In one capacity, PTH binds to its cognate receptor, PTH1R, a GPCR, on osteoblasts and enhances *Rankl* expression in a cAMP-dependent manner (Kondo et al. 2002). Consistent with this cAMP requirement, cell-based assays have suggested that CREB mediates the PTH regulation of *Rankl* expression (Fu et al. 2002). In vivo however, inactivation of *Creb* in osteoblasts does not affect *Rankl* expression or bone resorption (Kajimura et al. 2011) indicating that the transcriptional mechanisms whereby PTH signaling in osteoblasts affects *Rankl* expression may not be fully elucidated. Another systemic cue affecting *Rankl* expression in osteoblasts is the sympathetic nervous system. Catecholamines, following their binding to another GPCR, the β_2 adrenergic receptor, also use cAMP as a second messenger to enhance *Rankl* expression by recruiting the transcription factor ATF4 (Elefteriou et al., 2005). The sympathetic tone also influences through ATF4, the expression and activity of the hormone osteocalcin (Hinoi et al. 2008). These observations raise the following question: How do two distinct regulatory signals that use the same second messenger elicit different transcriptional events and genetic programs in the same cell type?

Chromatin structure, which is influenced by post-translational modifications of histone proteins around which the DNA is wrapped, is a major determinant of gene expression (Jenuwein and Allis 2001; Allis et al. 2006). Histone acetylation promotes gene transcription by relaxing the chromatin structure whereas deacetylation of histones by histone deacetylases (HDACs) induces chromatin condensation and transcriptional repression (Berger 2002; Verdin et al. 2003; Allis et al. 2006). A class of HDACs, the class II HDACs, besides containing a poorly active catalytic domain, has a long N-terminal extension to which transcription factors can bind. The existence of this domain has long suggested that class II

HDACs may serve as links between extracellular cues and the genome of a given cell (Verdin et al. 2003; Haberland et al. 2009). It is not known however, if and how a single class II HDAC molecule can integrate several extracellular cues to trigger different gene expression programs in the same cell.

One class II HDAC, HDAC4, prevents chondrocyte hypertrophy in part by interacting with and inhibiting in proliferating chondrocytes the activity of the transcription factor Runx2, a master gene of skeletogenesis (Vega et al. 2004). Given the fundamental functions that Runx2 exerts during osteoblast differentiation and as a regulator of bone formation, this observation raises the question of whether HDAC4 also regulates Runx2 functions in osteoblasts (Karsenty et al., 2009). In addressing the questions mentioned above, we show here that HDAC4 does not inhibit Runx2 functions in osteoblasts but rather integrates PTH and sympathetic signaling. HDAC4 inhibits *Rankl* expression in osteoblasts by interacting with MEF2c and preventing this transcription factor from transactivating the *Rankl* promoter. PTH signaling in osteoblasts favors *Rankl* expression by inducing, in part via the E3 ubiquitin ligase Smurf2, HDAC4 ubiquitination; this releases MEF2c that is now able to activate *Rankl* expression. In contrast, the sympathetic tone promotes HDAC4 accumulation in the nucleus of osteoblasts and its interaction with ATF4. Thus, under the control of the sympathetic tone, HDAC4 favors *Rankl* but also *Osteocalcin* expression and its endocrine and cognitive functions. We further show that HDAC4 does not inhibit Runx2 functions in osteoblasts because catecholamines disrupt the physical interaction between HDAC4 and Runx2. This study unravels the transcriptional bases of PTH regulation of *Rankl* expression in osteoblasts and identifies HDAC4 as a genomic integrator of two major regulatory signals in osteoblasts.

Results

HDAC4 inhibits *Rankl* expression in osteoblasts

With the goal of deciphering the functions of class II histone deacetylases (HDACs) in osteoblasts, we asked which ones were more highly expressed in these cells. *Hdac5* was, by far, the most highly expressed class II *Hdac* in osteoblasts (Figure 1A), its expression in bone being at least two-fold higher than in any other tissues tested (Figure 1B). *Hdac4* was the second highest expressed member of this gene family in osteoblasts; its expression was also higher in bone than in several other tissues like cartilage, brain and heart where this gene exerts important functions (Figure 1A-B) (Zhang et al. 2002; Chang et al. 2004; Vega et al. 2004). Similar results were obtained at the protein level (Figure 1C).

Given this pattern of expression, we focused our studies on HDAC4 and HDAC5 to elucidate the roles of class II HDACs in osteoblasts. For that purpose, we used *Hdac5-/-* mice and generated mice lacking *Hdac4* in cells of the osteoblast lineage to overcome the perinatal lethality of *Hdac4-/-* mice (Vega et al. 2004), This was achieved by crossing *Hdac4fl/fl* mice with mice expressing the *Cre* recombinase under the control of *Runx2* regulatory elements (Rauch et al. 2010). Prior to using $Hdac4_{osb}$*-/-* mice we verified that *Hdac4* had been efficiently deleted from osteoblasts but not from other cell types and that there was no overexpression of either *Hdac4* in the *Hdac5-/-* mice or of *Hdac5* in the $Hdac4_{osb}$*-/-* mice (Figure S1A, B). Since HDAC4 inhibits Runx2 functions in chondrocytes, we expected that it would do the same in osteoblasts and that $Hdac4_{osb}$*-/-* mice would display high bone mass because of increased osteoblast numbers and bone formation.

Instead, both $Hdac4_{osb}$*-/-* and *Hdac5-/-* mice demonstrated a low bone mass phenotype of similar intensity and affecting all bones analyzed (Figure 1D-E). This was caused by a 50% increase in the bone surface covered by osteoclasts compared to control mice whereas osteoblast numbers and bone formation rates were unaffected (Figure 1D). Accordingly, circulating levels of CTX, a byproduct of collagen degradation and a biomarker of bone resorption (Rosen et al. 2000), were significantly higher in $Hdac4_{osb}$*-/-* and *Hdac5-/-* than in control mice (Figure 1F). This increase in bone resorption was caused by a 2- to 10-fold increase in *Rankl* expression in *Hdac4-/-* and *Hdac5-/-* compared to control osteoblasts while *Opg* expression was not changed to the same extent (Figure 1G-H). As a result, the ratio of

Rankl/Opg was increased over 50% in *Hdac4-/-* and *Hdac5-/-* compared to WT osteoblasts (Figure 1I). Expression of other regulatory genes, such as *Runx2, Atf4, FoxO1, Mef2a* and *Mef2c* and of *type I collagen* was normal in *Hdac4-/-* or *Hdac5-/-* bones (Figure S1C). These results indicate that through their expression in osteoblasts, HDAC4 and HDAC5 inhibit *Rankl* expression and bone resorption but do not affect bone formation. An explanation for the absence of influence of HDAC4 or HDAC5 on bone formation is proposed below (see Figure 4H). For the rest of this study, because of the cell-specific nature of the gene deletion, we privileged the analysis of *Hdac4*$_{osb}$*-/-* mice.

HDAC4 inhibits osteoclast differentiation by preventing MEF2c to transactivate *RankI*

As a way to elucidate how HDAC4 inhibits *RankI* expression in osteoblasts, we asked whether members of the MEF2 family of transcription factors regulate *RankI* expression. This question was prompted by the documented interaction of HDAC4 with members of this family of transcription factors in other cell types (Zhang et al. 2002; Kozhemyakina et al. 2009), and by the presence in the mouse *RankI* promoter of 3 putative MEF2 binding sites that are conserved in all vertebrate species analyzed (Figure 2A).

Among the members of the *Mef2* gene family *Mef2a* and *Mef2c* were by far the most highly expressed in osteoblasts (Figure 2B). Chromatin immunoprecipitation assays verified that MEF2 protein could bind to each of the putative MEF2 binding sites present in the mouse *RankI* promoter (Figure 2C), and in DNA co-transfection assays performed in COS cells that do not express *RankI*, an expression vector for *Mef2c* transactivated a construct containing a 3kb-long fragment of the *RankI* promoter driving the *luciferase* gene (p*RankL-luc*) (Figure 2D). Disrupting each of these three binding sites decreased the activity of this promoter fragment by 50% or more and abrogated MEF2c ability to activate this *RankL-luc* construct (Figure 2D) thus illustrating the importance of MEF2 proteins for *RankI* expression in osteoblasts.

To assess the respective importance of MEF2a and MEF2c in the regulation of *RankI* in vivo, we generated mutant mice lacking either *Mef2a* or *Mef2c* in an osteoblast-specific manner (*Mef2a*$_{osb}$*-/-* and *Mef2c*$_{osb}$*-/-*). We verified that this manipulation had efficiently deleted *Mef2c* or *Mef2a* from osteoblasts but not from other cell types (Figure S2). Since our goal is to understand how HDAC4 regulates *RankI*

expression, we focused our analysis on *Rankl* expression and bone resorption. While mice lacking *Mef2a* in osteoblasts did not display any abnormalities of bone resorption, *Mef2c*$_{osb}$*-/-* mice exhibited a significant decrease in the bone surface covered by osteoclasts in all bones analyzed (Figure 2E). Serum CTX levels were also significantly lower in *Mef2c*$_{osb}$*-/-* than in *Mef2cfl/fl* mice and *Rankl* expression was decreased nearly 50% in *Mef2c*$_{osb}$*-/-* compared to control osteoblasts (Figure 2F-G). These results indicate that through its expression in osteoblasts, MEF2c is essential for *Rankl* expression in osteoblasts and osteoclast differentiation.

The opposite influence of HDAC4 and MEF2c on *Rankl* expression along with the ability of HDAC4 to interact physically with MEF2c prompted us to test whether HDAC4 inhibits *Rankl* expression by hampering the transactivating function of MEF2c. In DNA co-transfection experiments, the ability of MEF2c to transactivate the *RankL-luc* construct in COS cells was abrogated when either a *Hdac4* or a *Hdac5* expression vector was co-transfected (Figure 2H). More importantly in vivo, *Rankl* expression, serum CTx values as an indicator of bone resorption and bone mass were normal in *Hdac4*$_{osb}$*-/-* mice lacking one allele of *Mef2c* in osteoblasts only (*Hdac4*$_{osb}$*-/-; Mef2c*$_{osb}$*+/-*) (Figure 2I-L). Taken together the experiments presented above reveal the existence of an HDAC4 ⊣ MEF2c → *Rankl* regulatory loop taking place in osteoblasts in the mouse.

PTH favors *Rankl* expression through MEF2c by promoting HDAC4 proteasomal degradation

Given that the transcriptional means whereby PTH signaling in osteoblasts promotes *Rankl* expression are not completely understood, we asked whether PTH favors *Rankl* expression in osteoblasts, in part, by disrupting the HDAC4 ⊣ MEF2c → *Rankl* pathway.

Consistent with this hypothesis we observed that PTH (10nM) consistently increased the activity of the 3kb-long *Rankl* promoter fragment in ROS17/2.8 osteoblastic cells and that mutating the first MEF2 binding site in this promoter was sufficient to abrogate this effect of PTH (Figure 3A). Remarkably we also observed, whether this analysis was done by western blot or by immunofluorescence, that PTH triggers a marked decrease in HDAC4 accumulation in both the cytoplasm and the nucleus of mouse osteoblasts, while it did not decrease MEF2c accumulation in the nucleus of these cells (Figure 3B-D).

This was different from what is observed following treatment of chick chondrocytes with PTHrP, a molecule that bears similarities with PTH and that signals through the same receptor (Kronenberg 2003). Indeed, PTHrP allows HDAC4 to move to the nucleus by inducing its dephosphorylation on S246 (Kozhemyakina et al. 2009). PTH did induce this dephosphorylation event in HDAC4 in mouse osteoblasts (Figure 3E) but this could not explain our findings since PTH induces a near complete disappearance of HDAC4 from osteoblast nuclei (Figure 3C). Since PTH does not decrease *Hdac4* expression in osteoblasts (Figure S3A), we asked if instead PTH induces proteasomal degradation of HDAC4 in osteoblasts. In support of this idea, the PTH-induced decrease of HDAC4 accumulation in osteoblasts was prevented by the addition of an inhibitor of proteasomal degradation, bortezomib (Figure 3B-C). Bortezomib also significantly decreased the ability of PTH to up-regulate *Rankl* expression in osteoblasts (Figure 3F). Moreover, treating osteoblasts with PTH and bortezomib induced polyubiquitination of HDAC4 (Figure 3G). In view of these results, we asked whether PTH regulates the expression of any E3 ubiquitin ligases that are expressed in osteoblasts.

Among the E3 ubiquitin ligases that are highly expressed in osteoblasts (Severe et al. 2013), the expression of 2 of them, *Smurf2* and *Synv1* was significantly increased by PTH (Figure 3H). The regulation of *Smurf2* expression by PTH was specific since another extracellular signal, isoproterenol used as a surrogate of the sympathetic tone, did not affect *Smurf2* expression (Figure S3B). In view of these results, we studied the possible role of Smurf2 and Synv1 downstream of PTH signaling in osteoblasts through siRNA knockdown experiments. In each experiment we verified that we had efficiently decreased either *Smurf2* or *Synv1* accumulation, while not affecting *Hdac4* expression (Figure S3C). Decreasing Synv1 accumulation in osteoblasts increased *Rankl* expression and did not prevent PTH from inducing *Rankl* expression (Figure 3I). In contrast, decreasing Smurf2 accumulation in mouse osteoblasts significantly lowered the expression of *Rankl* and hampered the ability of PTH to induce *Rankl* expression (Figure 3I-J). Moreover, Smurf2 interacts with HDAC4 only if osteoblasts were treated with PTH (Figure 3K). In further support of a role of Smurf2 in the PTH regulation of *Rankl* expression, we observed that the PTH induction of *Rankl* expression was decreased nearly 50% in osteoblasts lacking one allele of *Ppr* encoding the PTH receptor and one allele of *Smurf2* (Figure 3L). Thus, our results suggest that one mechanism whereby PTH favors *Rankl* expression in osteoblasts is by recruiting Smurf2

to ubiquitinate HDAC4. This abrogates the well known physical interaction taking place between HDAC4 and MEF2c and allows MEF2c to activate the *Rankl* promoter (Backs et al. 2008).

The sympathetic tone favors *Rankl* expression by stabilizing HDAC4

The observation that PTH regulates *Rankl* expression in an HDAC4-dependent manner raised the hypothesis that another systemic regulator of *Rankl* expression in osteoblasts, the sympathetic nervous system (Elefteriou et al. 2005), might also recruit HDAC4 to achieve this function.

To test if it was the case, we treated mouse osteoblasts with isoproterenol (ISO) (10μM), a surrogate of the sympathetic tone. Like PTH, ISO favored *Hdac4* and to a lesser extent *Hdac5* expression (Figure 4A). However unlike PTH, ISO increased HDAC4 accumulation in the nucleus of osteoblasts and promoted the interaction of this class II HDAC with ATF4 (Figure 4B-C). ISO also decreased the phosphorylation of HDAC4 on S246 an event that favors translocation of HDAC4 to the nucleus (Figure 4D). Accordingly, mice lacking one allele of *Hdac4* and one allele of *Atf4* only in osteoblasts ($Atf4_{osb}$*+/-;* $Hdac4_{osb}$*+/-* mice) demonstrated a significant decrease in Rankl expression, circulating CTx values and the bone surface covered by osteoclasts (Figure 4E-G). Thus, both PTH and the sympathetic tone target HDAC4 to promote *Rankl* expression but they do so differently. PTH favors HDAC4 degradation to allow MEF2c to increase *Rankl* expression; the sympathetic tone instead enhances HDAC4 accumulation to favor its interaction with ATF4 and *Rankl* expression.

Of note, while it increased the interaction between HDAC4 and ATF4, ISO treatment of ROS17/2.8 osteoblastic cells disrupted the interaction between HDAC4 or 5 and Runx2. Similar results were obtained when using mouse primary osteoblasts (Figure 4H). This latter observation is important as it provides an explanation for why in the living animal in which the sympathetic signaling is constantly present, disruption of HDAC4 in osteoblasts does not affect Runx2-regulated bone formation (Ducy et al., 1999).

HDAC4 regulates osteocalcin endocrine and cognitive functions through its expression in osteoblasts.

Osteoblasts are also endocrine cells. Since ATF4 regulates the expression of one of the osteoblast-derived hormones, *Osteocalcin* (Yang et al. 2004), the interaction between ATF4 and HDAC4 presented above raised the prospect that HDAC4 might also affect the endocrine and cognitive functions mediated by osteocalcin.

Expression of *Osteocalcin* as well as of *Esp*, a gene encoding an inhibitor of osteocalcin activity (Lee et al. 2007), was decreased in $Hdac4_{osb}$*-/-* and in $Atf4_{osb}$*+/-;* $Hdac4_{osb}$*+/-* but not in *Hdac5-/-* bones thus revealing one functional difference between these two class II HDACs (Figure 5A). This feature of $Hdac4_{osb}$*-/-* mice was expected since ATF4, with whom HDAC4 interacts, regulates the expression of both genes (Yang et al. 2004; Yoshizawa et al. 2009). Circulating levels of total and undercarboxylated i.e., active, osteocalcin were decreased in $Hdac4_{osb}$*-/-* and $Atf4_{osb}$*+/-;*$Hdac4_{osb}$*+/-* mice (Figure 5B). This decrease, however, was less severe than one would have anticipated given the marked decrease in *Osteocalcin* expression in $Hdac4_{osb}$*-/-* bones (Figure 5A). There may be two complementary explanations for this. First, the decreased expression of *Esp*, a negative regulator of osteocalcin bioactivity (Lee et al. 2007), should oppose the decrease in the circulating levels of active osteocalcin; second, the increase in bone resorption, a positive regulator of osteocalcin undercarboxylation (Ferron et al. 2010), seen in $Hdac4_{osb}$*-/-* mice should also counteract the effect of the decrease in *Osteocalcin* expression on the circulating levels of active osteocalcin in $Hdac4_{osb}$*-/-* mice. Nevertheless, osteocalcin circulating levels were significantly decreased in $Hdac4_{osb}$*-/-* mice, an observation that prompted us to analyze their endocrine and cognitive functions.

Circulating levels of insulin were decreased in both $Hdac4_{osb}$*-/-* and in $Atf4_{osb}$*+/-;*$Hdac4_{osb}$*+/-* mice (Figure 5C-D), moreover, $Hdac4_{osb}$*-/-* mice displayed a decrease in insulin secretion as measured by a glucose-stimulated insulin secretion (GSIS) test (Figure 5E), and a significant decrease in the β-cell area and in insulin content in their pancreata (Figure 5F-G). That insulin sensitivity was not affected in $Hdac4_{osb}$*-/-* mice (Figure S4) is consistent with the relatively mild decrease in the circulating levels of undercarboxylated osteocalcin since we know that this function of osteocalcin requires a higher threshold of signaling to be affected (Ferron et al. 2008). Osteocalcin also favors male fertility by signaling in cells of

the testes (Oury et al. 2011). Accordingly testes size and weight, epididymes and seminal vesicle weights, sperm count and testosterone levels were all decreased in *Hdac4$_{osb}$-/-* mice (Figure 5H-J). Lastly, osteocalcin favors spatial learning and memory, and these two functions are hampered in *Osteocalcin*+/- mice (Oury et al. 2013). In a Morris Water Maze test (MWMT) assessing spatial learning and memory through the ability of mice to find a submerged platform using spatial cues (Morris et al. 1982), *Hdac4$_{osb}$-/-* mice performed as poorly as *Osteocalcin+/-* mice (Oury et al. 2013b) (Figure 5K). Thus, through its association with ATF4, HDAC4 also regulates the endocrine and cognitive functions of osteocalcin.

Discussion

This study, initiated to better understand the functions of class II HDACs in osteoblasts, revealed several novel molecular aspects of osteoblast biology. It identified a transcriptional mediator of PTH signaling in osteoblasts and showed that HDAC4 and HDAC5 integrate, in different ways, two extracellular cues, PTH and the sympathetic tone. This triggers different transcriptional events in osteoblasts that result in the enhancement of *Rankl* expression and of the endocrine and cognitive functions of osteoblasts. Lastly, it provides an explanation for why HDAC4 does not inhibit Runx2 function in osteoblasts as it does in chondrocytes.

Class II HDACs expression and functions in osteoblasts

The role of HDAC4 as an inhibitor of Runx2 functions in proliferating chondrocytes (Vega et al. 2004), begged the question of the role that this or any other class II HDACs might have in osteoblasts, a cell type where Runx2 is so important before and after birth (Ducy et al. 1997; Komori et al. 1997; Otto et al. 1997; Ducy et al. 1999). Two class II HDACs, HDAC4 and HDAC5, are highly expressed in osteoblasts. To our surprise however, deletion of either one of these 2 class II HDACs in osteoblasts does not result in the expected increase in Runx2 function and therefore in an increase in bone formation and bone mass. Instead both *Hdac5-/-* and *Hdac4$_{osb}$-/-* mice display a low bone mass because of an isolated increase in bone resorption parameters. Further molecular and genetic tests showed that through their expression in osteoblasts *Hdac4* and *Hdac5* prevent expression of *Rankl,* the major osteoclast

differentiation factor (Teitelbaum, 2000). They do so because through their interaction with MEF2c, they prevent this transcription factor to activate the *Rankl* promoter (Figure 6). These results show that class II HDACs have different modes of action in two related skeletal cell types, chondrocytes and osteoblasts. Since Runx2 is expressed in osteocytes (Ducy et al. 1999), the *Cre*-expressing mouse used in this study should delete genes equally well in osteoblasts and osteocytes (Rauch et al. 2010). Therefore, we anticipate that the mechanism of action described in this study may be at work in osteocytes where *Rankl* expression is so high (Nakashima et al. 2011; Xiong et al. 2011). Further experiments using an osteocyte-specific Cre deleting mouse will address this question.

PTH regulation of *Rankl* expression

The notion that class II HDACs serve as links between extracellular cues and the genome of a given cell, together with the uncertainties regarding the transcriptional mode of action of PTH in osteoblasts led us to ask whether PTH signaling in osteoblasts interferes with the regulatory loop mentioned above to favor *Rankl* expression.

Several lines of evidence indicate that it is the case and that PTH does so through a different mechanism than the one used by its close relative, PTHrP, in chick chondrocytes (Kozhemyakina et al. 2009). Indeed, PTH treatment of osteoblasts causes a near disappearance of HDAC4 away from any compartment in osteoblasts, a phenomenon blocked by inhibiting proteasomal degradation, whereas PTHrP treatment of chick chondrocytes causes an accumulation of HDAC4 in the nucleus (Kozhemyakina et al. 2009). Accordingly for these differences PTH enhances expression in osteoblasts of at least one E3 ubiquitin ligase, *Smurf2,* and is necessary for the interaction of Smurf2 and HDAC4 to occur. Accordingly, PTH triggers a Smurf2-dependent degradation of HDAC4 and the PTH ability to induce *Rankl* expression is diminished by decreasing Smurf2 accumulation in osteoblasts and in *Ppr+/-;Smurf2+/-* osteoblasts. Further genetic evidence is now needed to determine the in vivo importance of Smurf2 in the ubiquitination of HDAC4 in osteoblasts. Our results do not rule out that other E3 ubiquitin ligases, not identified in this study, contribute to the PTH-mediated ubiquitination of HDAC4. Finally we should also emphasize that our results do not contradict and do not exclude in any way the fact that PTH

also favors *Rankl* expression in osteoblasts by signaling in T-lymphocytes as it has been demonstrated (Gao et al. 2008; Tawfeek et al. 2010).

Sympathetic signaling in osteoblasts and class II HDACs

PTH is not the only extracellular cue recruiting HDAC4 in osteoblasts to mediate its functions. Our results show that the sympathetic tone does the same although it exerts a totally different influence than PTH on HDAC4 accumulation.

Like PTH, the sympathetic tone favors *Hdac4* and *Hdac5* expression but unlike PTH, the sympathetic tone favors the accumulation of HDAC4 in osteoblasts and its interaction with ATF4, the only known transcriptional mediator of the sympathetic regulation of *Rankl* expression (Elefteriou et al. 2005). The biological importance of the HDAC4-ATF4 interaction was demonstrated in vivo by showing that $Atf4_{osb}$+/-;$Hdac4_{osb}$+/- mice demonstrate a decrease in *Rankl* expression and in bone surface covered by osteoclasts. This mechanism is different from the PKA-induced proteolysis described by Backs et al., in cardiomyocytes (Backs et al. 2011). Although we cannot exclude that such a proteolysis occurs in osteoblasts, we note that if it were the case it would prevent expression of *Rankl* whereas ISO increases it. The different mechanisms whereby PTH and the sympathetic tone use HDAC4 as an intermediary step to fulfill their functions in osteoblasts (Figure 6) suggest that these two extracellular cues recruit different signaling molecules. Further biochemical studies will help clarify this aspect of PTH and sympathetic signaling in osteoblasts.

Sympathetic signaling, class II HDACs and Runx2 function in osteoblasts

HDAC4 importance during skeletogenesis first came to light when it was shown that it interacts physically with Runx2 to inhibit the transactivating function of this factor in proliferating chondrocytes, and therefore chondrocyte hypertrophy (Vega et al. 2004). Given these results, it came as a surprise that neither HDAC4 nor HDAC5 deletion in osteoblasts affected osteoblast numbers and bone formation, two classical functions of Runx2 (Ducy et al. 1997; Komori et al. 1997; Karsenty et al. 1999). Our experiments indicate that while it favors ATF4 interaction with HDAC4 and HDAC5, catecholamines inhibit the physical interaction between HDAC4 or HDAC5 and Runx2 in osteoblasts. Since catecholamines are constantly

present in bones, which are vascularized and highly innervated tissues (Chenu 2004; Lafage-Proust et al. 2010), this observation provides a plausible explanation for the fact that HDAC4 and HDAC5 deletions in osteoblasts do not affect osteoblasts number and bone function. Since cartilage is avascular and poorly innervated if at all the interaction between HDAC4 and Runx2 can occur in chondrocytes (Vega et al., 2004). This finding also underscores the physiological importance of sympathetic signaling in osteoblasts in determining bone mass accrual (Takeda et al. 2002; Elefteriou et al. 2005).

Hdac4 expression in osteoblasts and endocrine and cognitive functions of the osteoblasts

The central role that HDAC4 and HDAC5 play as partners of ATF4 begged the question of their involvement in regulating the functions of osteocalcin, a hormone made by osteoblasts and whose expression is regulated by ATF4 (Ducy and Karsenty 1995; Yang et al. 2004). This is an important question to address if one wants to achieve a better molecular understanding of the emerging role of the osteoblasts as a multipurpose cell type (Karsenty and Ferron 2012). Our studies show that, in cell culture like in vivo, HDAC4 but not HDAC5 regulates osteocalcin expression, accumulation and bioactivity. As a result and through its expression in osteoblasts, HDAC4 favors insulin secretion, glucose homeostasis, male fertility and spatial learning and memory. The fact that HDAC5 does not fulfill the same function than HDAC4 in this setting suggests that each class II HDAC might interact with different factors. In a broader sense, the results presented here underscore the importance of HDAC4 compared to other class II HDACs as a global regulator of several functions of the osteoblast (Figure 6), and are an incentive to look for signaling molecules expressed in osteoblasts and interacting preferentially with HDAC4.

Experimental Procedures

Mouse Generation

For all the in vivo analysis except for the Morris Water Maze test male mice of indicated ages were used. In the Morris Water Maze test, female mice of 3-month of age were used. Generation of *Hdac5-/-* mice was previously reported (Chang et al. 2004). *Hdac4*$_{osb}$*-/-* mice were generated by intercrossing *Hdac4fl/fl* mice with *Runx2-Cre* transgenic mice (Rauch et al. 2010). *Mef2a*$_{osb}$*-/-* and *Mef2c*$_{osb}$*-/-* mice (C57BL/6J) were generated by intercrossing *Mef2afl/fl* or *Mef2cfl/fl* mice (Lin et al. 1997;

Akhtar et al. 2012) and *Runx2-Cre* transgenic mice. *Hdac4$_{osb}$-/-;Mef2c$_{osb}$+/-* mice were obtained from crosses between F2 generation *Hdac4$_{osb}$ +/-;Mef2c$_{osb}$+/-* and *Hdac4fl/fl* or *Hdac4$_{osb}$+/-* and *Hdac4$_{osb}$+/-;Mef2c$_{osb}$+/-* mice. *Hdac4$_{osb}$+/-;Atf4$_{osb}$+/-* mice were generated by crossing *Atf4fl/fl* or *Atf4fl/+* mice with *Hdac4$_{osb}$+/-* mice. Mice genotypes were determined by PCR (primer sequences available upon request). Floxed mice and *Cre* expressing mice were used as controls. All animal procedures were approved by CUMC IACUC and conform to the relevant regulatory standards.

Bone histomorphometry

Lumbar vertebrae or tibia dissected from 2- or 3- month old mice were fixed for 24 hours, dehydrated with graded concentrations of ethanol and embedded in methyl methacrylate (MMA) resin according to standard protocols. Von Kossa/Van Gieson, toluidine blue and tartrate-resistant acid phosphatase (TRAP) stainings were used to measure bone volume over tissue volume (BV/TV), osteoblast and osteoclast numbers and surface, respectively. Bone formation rates were assessed following calcein double-labeling. Calcein (Sigma Chemical Co., St. Louis, MO) was dissolved in calcein buffer (0.15 M NaCl, 2% NaHCO3) and injected twice at 0.125 mg/g body weight on days 1 and 4, and mice were killed on day 6. All quantifications were performed using Osteomeasure Analysis system (Osteometrics, Atlanta GA).

Metabolic Studies and Bioassays

For insulin tolerance test (ITT), mice were fasted for 4 hours, and then insulin (0.44U/kg) was given with intraperitoneal injection (IP). Blood glucose levels were measured at indicated time points using the Accu-Chek active system (Roche). For glucose-stimulated insulin secretion (GSIS) test, mice were fasted for 16 h an injected IP with 3g/kg of glucose. Blood was collected from the tail of the mice at indicated time points and serum insulin levels were assessed using the Ultra-sensitive Insulin ELISA kit (Crystal Chem). Random insulin levels in mice were measured in tail blood collected and assayed with the mouse/human insulin ELISA (Mercodia). Pancreas insulin content and histology was performed as previously described (Lee et al. 2007; Ferron et al. 2010). CTX were measured using the RatLaps ELISA (ImmunoDiagnostics). Total and undercarboxylated osteocalcin levels were obtained using a previously

described ELISA (Ferron et al. 2010) Serum testosterone levels were measured by radioactive immunoassay (Alpco).

Cell-based assays

Mouse osteoblasts were prepared as previously described (Ducy and Karsenty 1995; Ferron et al. 2010). For in vitro gene inactivation, floxed osteoblasts were divided into two groups and infected with either GFP- or Cre-expressing adenovirus (University of Iowa). For siRNA transfections, osteoblasts were transfected with siRNA pools (On-TARGETplus, Dharmacon) according to the manufacturer instructions. For immunofluorescence cells were trypsinized, re-plated after two days of culture and left in regular media conditions for one day. On experiment day, cells were washed twice with PBS and treated with indicated amount of PTH (1-34) (PROSPEC), ISO (Sigma) and bortezomib (Selleckchem) for 2 hours. Immunofluorescence was performed using a standard protocol. For gene expression, osteoblasts were cultured in α-MEM containing 10% FBS supplemented with 5 mM β-glycerophosphate and 100 µg/ml ascorbic acid for 10 days. Cells were fasted in serum-free media for 16 hours and treated with indicated amount of PTH or ISO or bortezomib for 2 hours. Cells were then collected in TRizol (Invitrogen), RNA isolation, cDNA preparation, and qPCR analyses were carried out following standard protocols.

Molecular biology and Biochemistry

For DNA co-transfection assays COS cells were cultured in DMEM supplemented with 10% FBS. Cells were transfected with pCMV14-FLAG-MEF2c (100ng), pCDNA3.1-HA-HDAC4 (10ng) or pCDNA3.1-HA-HDAC5 (10ng) expression vectors, *pRankl-luc*, *pRankls1-luc, pRankls2-luc* or *pRankls3-luc* (5ng) and *pCMV-β-gal* reporter vectors. DNA transfections were performed in ROS17/2.8 cells cultured in DMEM F12 (1:1) supplemented with 10% FBS. Cells were transfected with *pRankl-luc* (5ng) or *pRankls1-luc* (5ng) and *pCMV-β-gal* (200ng) reporter vectors. Thirty-two hours after transfection cells were washed with PBS and treated with serum-free media containing either 10nM PTH or vehicle for indicated times. Luciferase and β-gal assays were performed using standard procedures. For co-immunoprecipitation

assays, ROS17/2.8 cells were transfected with pCMV5-FLAG-ATF4 (6 μg), pCMV5-FLAG-Runx2 (6 μg), pCMV5-Smurf2 (6ug) with either pCDNA3.1-HA-HDAC4 (2 μg) or pCDNA3.1-HA-HDAC5 (2 μg). Forty-eight hours later cells were washed with PBS and treated with media containing 0.1% FBS and indicated amount of PTH or Bortezomib or ISO for 2 hours. Cells were lysed in hypotonic buffer and disrupted by Dounce homogenizer. The cytosolic fraction was separated from the pellet by centrifugation at 4°C. The nuclear-soluble fraction was obtained by incubation of the pellet in high-salt buffer to get a final NaCl concentration of 300mM. Tagged proteins from each fraction were immunoprecipitated with anti-Flag M2 overnight. The next day protein G-agarose beads were added and incubated for 2 hours at 4°C. Each IP was washed 5 times with TGEN 150 buffer (20mM Tris at pH 7.65, 150mM NaCl, 3mM MgCl2, 0.1mM EDTA, 10% glycerol, 0 0.01% NP40), proteins were eluted with Laemmli buffer and boiled for 10 minutes.

Antibodies used in this study are anti-FLAG (Sigma), anti-MEF2c (Abcam,), anti-MEF2 (Santa Cruz Biotechnology), anti-HDAC4 (Santa Cruz Biotechnology), anti-HA (Cell Signaling Technology), anti-HDAC5 (Cell Signaling Technology), anti-phospho-HDAC4 S-246 (Cohen et al. 2007), anti-GAPDH (Cell Signaling Technology), and anti-ubiquitin (Cell Signaling Technology). DAPI was included in the mounting media (EMS).

Chromatin immunoprecipitation

Mouse osteoblasts were washed twice with PBS and cross-linked with 1.1% formaldehyde for 10 minutes at 37°C. Crosslinking was stopped by 125mM glycine with gentle rocking. Cells were washed with PBS containing 0.25mM PMSF, nuclear extracts prepared and each sample sonicated to obtain sheared fragments of 200-800 bp. Samples were pre-cleared with normal rabbit IgG with incubation for 1 hour and agarose G beads for 2 hours at 4°C. An anti-MEF2 antibody was added to cleared lysates and incubated overnight at 4°C. The next day, agarose protein G beads were blocked with 200 μg/ml salmon sperm DNA and 1mg/ml BSA for 1 hour at 4°C. Lysates were incubated with blocked beads for 2 hours at 4°C, washed in low salt buffer, twice in high salt, twice in LiCl buffer and twice in TE buffer. Washed beads were eluted in elution buffer by incubating at 37°C for 2 hours. The DNA was reverse cross-linked by incubating all samples at 65°C for 6 hours. Following DNA extraction, PCR analysis was performed on ChIPed material with oligonucleotides designed to detect the Mef2 binding sites within the mouse *Rankl*

promoter.

Morris Water Maze test

The test was performed on female mice only and as previously described (Oury et al., 2013).

Statistical analysis

Results are given as means ± standard error of the mean (SEM). Statistical analyses were performed using unpaired, two-tailed Student's t-test. In all figures error bars represent SEM and * represents $p<0.05$.

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Figure 1. HDAC4 and HDAC5 inhibit *Rankl* expression in osteoblasts

(A) Analysis of Class IIa *Hdacs* expression in WT osteoblasts (n=3). The expression of each gene is compared to the one of *Hdac5.* (B) Analysis of *Hdac4* (right panel) and *Hdac5* (left panel) expression in various tissues (n=3). Expression of these 2 genes in each tissue is compared to their expression in muscle. (C) Western blot analysis of HDAC4 and HDAC5 accumulation in different tissues. Anti-GAPDH was used as a loading control. (D) Histomorphometric analyses of vertebrae of 3 month-old *Hdac4*$_{osb}$*-/-* (n=7) and *Hdac5-/-* (n=7) mice compared to *Hdac4fl/fl* (n=8) or WT (n=5) controls respectively. BV/TV%: percentage of bone volume over trabecular volume; Nb.Ob/T.Ar.: number of osteoblasts per trabecular area; BFR: bone formation rate; OcS/BS%: percentage of osteoclast surface per bone surface. (E) Histomorphometric analyses of long bones of 3 month-old *Hdac4fl/fl* (n=6), *Hdac4*$_{osb}$*-/-* (n=5), WT (n=5) and *Hdac5-/-* (n=6). (F) Serum CTX levels in 3 months-old *Hdac4fl/fl* (n=13), *Hdac4* $_{osb}$*-/-* (n=12), WT (n=10), *Hdac5-/-* (n=9). Results are expressed as fold changes compared to levels seen in *Hdac4fl/fl* mice. (G) Analysis of *Rankl* expression in *Hdac4fl/fl* (n=4), *Hdac4-/-* (n=4) (left panel), WT(n=4) and *Hdac5-/-* (n=4) (right panel) osteoblasts. Results are presented as fold changes compared to levels seen in *Hdac4fl/fl* or WT osteoblasts. (H, I) Analysis of *Opg* expression (H) and *Rankl/Opg* ratio (I) in *Hdac4fl/fl* (n=4), *Hdac4-/-* (n=4), WT (n=4) and *Hdac5-/-* (n=4) osteoblasts. Results are expressed as fold changes compared to levels seen in *Hdac4fl/fl* or WT osteoblasts. Results are given as means ± SEM. *p<0.05 by Student's test.

Figure 1

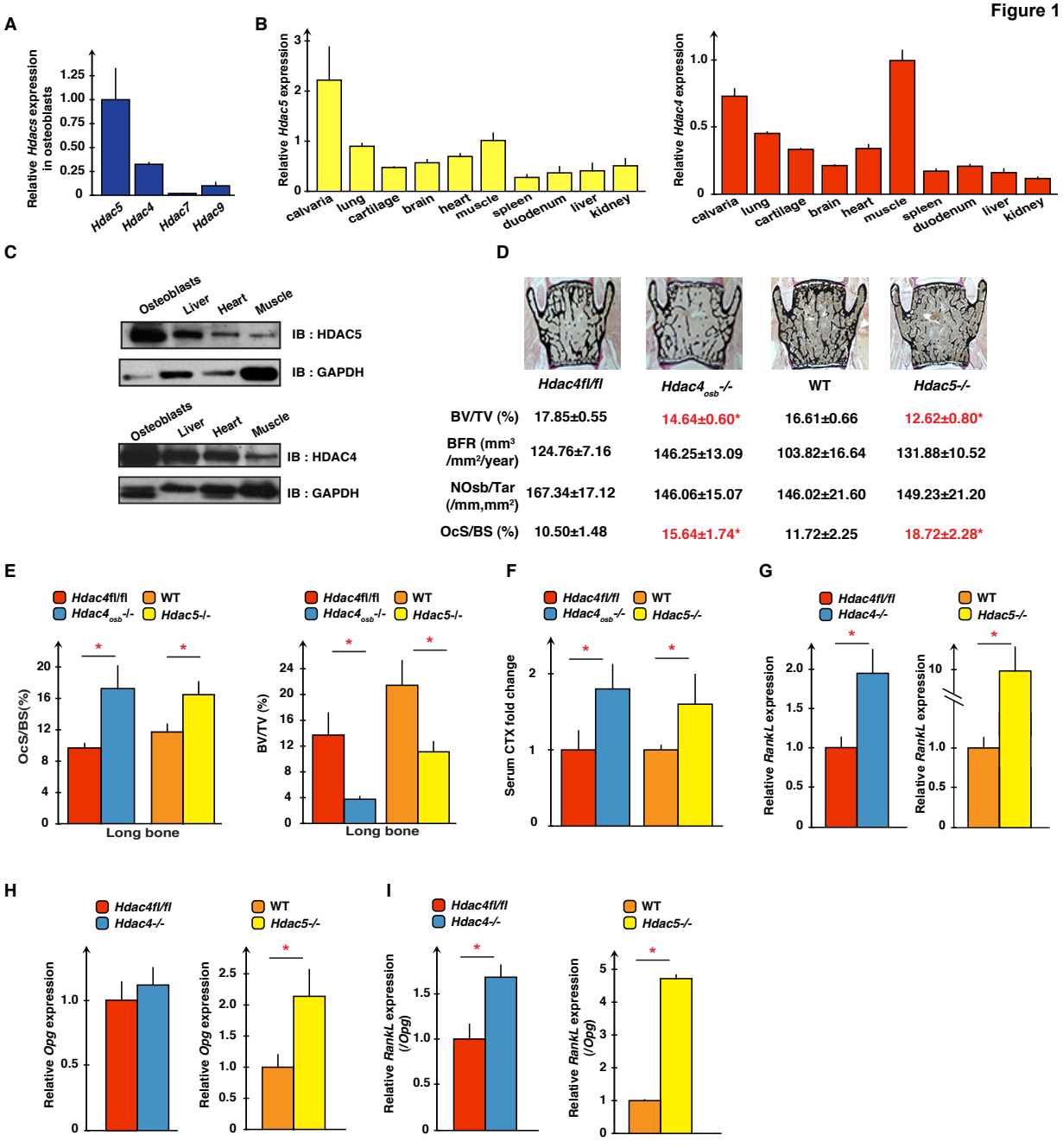

A
Relative Hdacs expression in osteoblasts
Hdac5
Hdac4
Hdac7
Hdac9
B
Relative Hdac5 expression
calvaria
lung
cartilage
brain
heart
muscle
spleen
duodenum
liver
kidney
Relative Hdac4 expression
C
Osteoblasts
Liver
Heart
Muscle
IB : HDAC5
IB : GAPDH
IB : HDAC4
IB : GAPDH
D
Hdac4fl/fl
Hdac4osb-/-
WT
Hdac5-/-
BV/TV (%) 17.85±0.55 14.64±0.60* 16.61±0.66 12.62±0.80*
BFR (mm3 /mm2/year) 124.76±7.16 146.25±13.09 103.82±16.64 131.88±10.52
NOsb/Tar (/mm,mm2) 167.34±17.12 146.06±15.07 146.02±21.60 149.23±21.20
OcS/BS (%) 10.50±1.48 15.64±1.74* 11.72±2.25 18.72±2.28*
E
Hdac4fl/fl
Hdac4osb-/-
WT
Hdac5-/-
OcS/BS(%)
Long bone
BV/TV (%)
Long bone
F
Serum CTX fold change
G
Hdac4fl/fl
Hdac4-/-
WT
Hdac5-/-
Relative RankL expression
H
Relative Opg expression
I
Relative RankL expression (/Opg)

Figure 2. HDAC4 prevents MEF2c to transactivate *Rankl*

(A) Putative MEF2 binding sites in the *Rankl* promoter region of the mouse and other bony vertebrate species. (B) *Mef2* genes expression in WT osteoblasts (n=3). Expression of each gene is compared to the one of *Mef2a*. (C) Chromatin immunoprecipitation (ChIP) assay in mouse osteoblasts analyzing MEF2 binding to the *Rankl* promoter. (D) DNA co-transfection assays in COS cells with a *Rankl* promoter luciferase construct (p*RankL-luc*) either WT or harboring a mutation in either one of the MEF2 binding sites alone or with a MEF2c expression vector. (E) Histomorphometric quantification of the bone surface covered by osteoclasts in vertebrae of 2 month-old *Mef2afl/fl* (n=5), *Mef2a*$_{osb}$*-/-* (n=5), *Mef2cfl/fl* (n=6) and *Mef2c*$_{osb}$*-/-* (n=8) (left panel) mice and long bones of *Mef2cfl/fl* (n=6) and *Mef2c*$_{osb}$*-/-* (n=8) mice (right panel). (F) Serum CTX levels in 2 month-old *Mef2afl/fl* (n=8), *Mef2a*$_{osb}$*-/-* (n=7), *Mef2cfl/fl* (n=6) and *Mef2c*$_{osb}$*-/-* (n=7) mice. Results are represented as fold changes compared to levels seen in *Mef2cfl/fl* mice. (G) Analysis of *Rankl* expression in *Mef2afl/fl* (n=8), *Mef2a*$_{osb}$*-/-* (n=7), *Mef2cfl/fl* (n=13) and *Mef2c*$_{osb}$*-/-* (n=14) mice. Results are expressed as fold changes compared to levels seen in *Mef2cfl/fl* mice. (H) DNA co-transfection assays in COS cells of a *Rankl* promoter luciferase construct (p*RankL-luc*) either WT or mutant for the MEF2 binding site 1, along with MEF2c and HDAC4 or HDAC5 expression vectors. (I) Histomorphometric quantification of the bone surface covered by osteoclasts in the vertebrae of 2 month-old control (n=5), *Mef2c*$_{osb}$*+/-* (n=4), *Hdac4*$_{osb}$*-/-* (n=4) and *Hdac4*$_{osb}$*-/-*;*Mef2c*$_{osb}$+/- (n=3) mice. (J) Analysis of *Rankl* expression in control (n=6), *Mef2c*$_{osb}$*+/-* (n=3), *Hdac4*$_{osb}$*-/-* (n=4) and *Hdac4*$_{osb}$*-/-* ;*Mef2c*$_{osb}$+/- (n=3) calvaria. Results are presented as fold changes compared to levels seen in WT osteoblasts. (K) Serum CTX levels of control (n=6), *Mef2c*$_{osb}$*+/-* (n=3), *Hdac4*$_{osb}$*-/-* (n=4) and *Hdac4*$_{osb}$*-/-* ;*Mef2c*$_{osb}$+/- (n=3) mice. Results are presented as fold changes compared to levels seen in WT mice. (L) Histomorphometric analyses of BV/TV% (percentage of bone volume over trabecular volume) in of vertebrae 2 month-old control (n=9), *Mef2c*$_{osb}$*+/-* (n=4), *Hdac4*$_{osb}$*-/-* (n=5) and *Hdac4*$_{osb}$*-/-*;*Mef2c*$_{osb}$+/- (n=3) mice. Results are given as means ± SEM. *p<0.05 by Student's test.

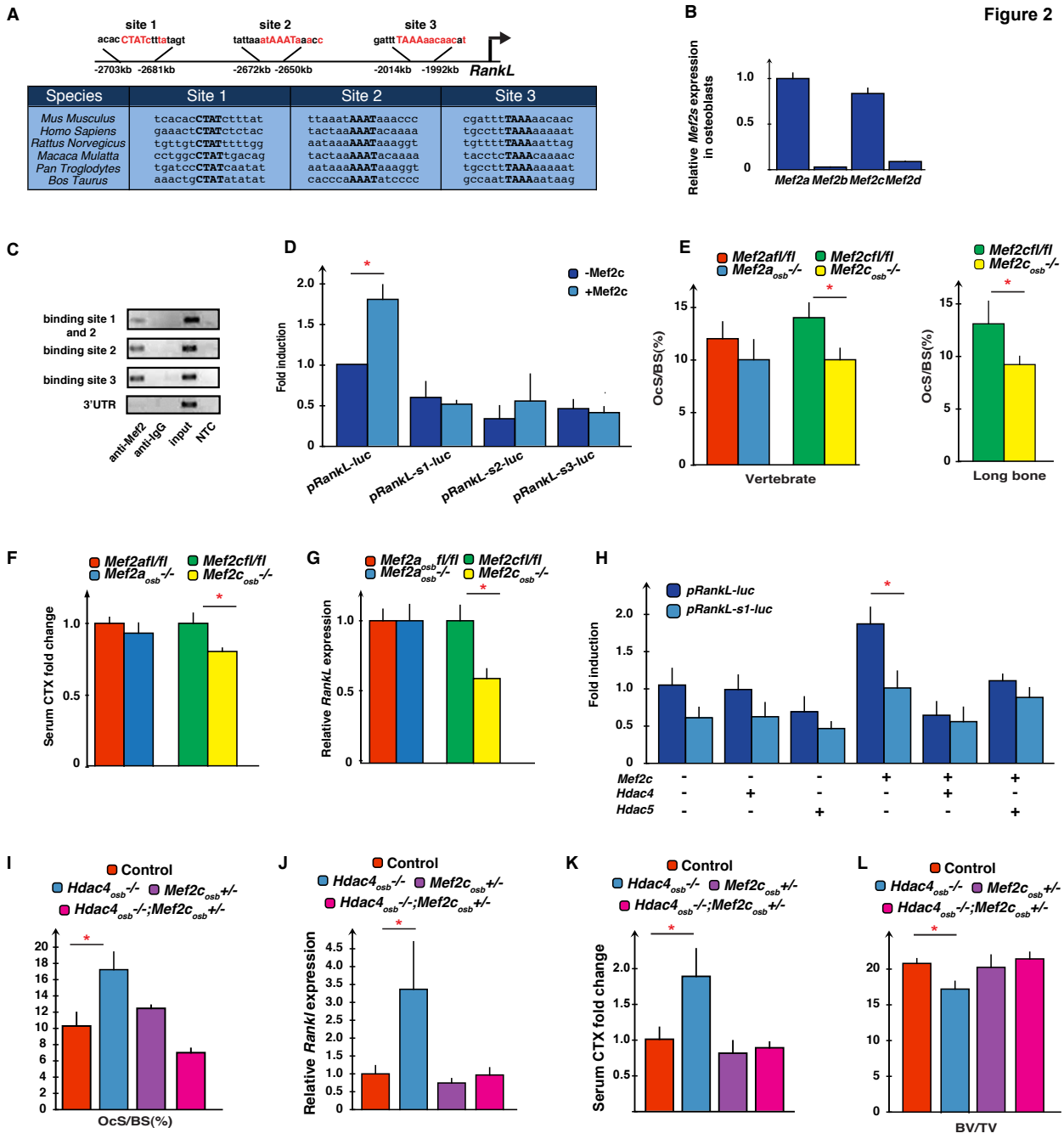


Figure 2
A
site 1
site 2
site 3
acac CTATctttatagt
tattaaatAAATaaacc
gattt TAAAaacaacat
-2703kb -2681kb
-2672kb -2650kb
-2014kb -1992kb
RankL
Species
Site 1
Site 2
Site 3
Mus Musculus
Homo Sapiens
Rattus Norvegicus
Macaca Mulatta
Pan Troglodytes
Bos Taurus
tcacacCTATctttat
gaaactCTATctctac
tgttgtCTATttttgg
cctggcCTATtgacag
tgatccCTATcaatat
aaactgCTATatatat
ttaaatAAATaaaccc
tactaaAAATacaaaa
aataaaAAATaaaggt
tactaaAAATacaaaa
aataaaAAATaaaggt
cacccaAAATatcccc
cgatttTAAAaacaac
tgccttTAAAaaaaat
tgttttTAAAaattag
tacctcTAAAcaaaac
tgccttTAAAaaaaat
gccaatTAAAaataag
B
Relative Mef2s expression in osteoblasts
1.0
0.5
0
Mef2a Mef2b Mef2c Mef2d
C
binding site 1 and 2
binding site 2
binding site 3
3'UTR
anti-Mef2
anti-IgG
input
NTC
D
-Mef2c
+Mef2c
Fold induction
pRankL-luc
pRankL-s1-luc
pRankL-s2-luc
pRankL-s3-luc
E
Mef2afl/fl
Mef2a osb -/-
Mef2cfl/fl
Mef2c osb -/-
OcS/BS(%)
Vertebrate
Long bone
F
Serum CTX fold change
G
Relative RankL expression
H
pRankL-luc
pRankL-s1-luc
Fold induction
Mef2c
Hdac4
Hdac5
I
Control
Hdac4 osb -/-
Mef2c osb +/-
Hdac4 osb -/-;Mef2c osb +/-
OcS/BS(%)
J
Relative Rankl expression
K
Serum CTX fold change
L
BV/TV

Figure 3. PTH favors *Rankl* expression by disrupting the HDAC4-MEF2c interaction.

(A) DNA transfection assays in ROS17/2.8 cells using a p*Rankl*-luc reporter construct either WT (*pRankL-luc*) or mutated for the MEF2 binding site 1 (*pRankLs1-luc*). Cells were treated with PTH (10nM) or vehicle for 16 hours. (B) Western blot analysis of total extracts from osteoblasts treated with vehicle, PTH (10nM) or PTH plus bortezomib (25nM) for 2 hours using anti-HDAC4 and anti-βactin antibodies. (C) Immunofluorescence showing HDAC4 localization in mouse osteoblasts treated with vehicle or PTH (10nM) alone or in the presence of bortezomib (25nM) for 2 hours. (D) Immunofluorescence assays showing MEF2C localization in mouse osteoblasts treated with vehicle or PTH (10nM) for 2 hours. (E) Immunoprecipitation of HDAC4 in WT osteoblasts treated with vehicle or PTH (10nM) plus bortezomib (25nM). Proteins were analyzed by Western Blot using anti-HDAC4 or anti-HDAC4phosphoS246 antibodies. (F) Analysis of *Rankl* expression in WT osteoblasts treated with vehicle, PTH (10nM) or PTH plus bortezomib (100nM) for 2 hours. Results are presented as fold changes compared to levels seen in vehicle treated osteoblasts. (G) Immunoprecipitation of HDAC4 in WT osteoblasts treated with vehicle or PTH plus bortezomib (25nM) for 2 hours. Proteins were analyzed by Western Blot using anti-HDAC4 or anti-ubiquitin antibodies. (H) Analysis of expression of E3 ubiquitin ligases in WT osteoblasts treated with PTH (10nM) for 2 hours. Results for each gene is presented as fold change compared to levels seen in vehicle treated osteoblasts. (I) Analysis of *Rankl* expression in scrambled, *Synv1* or *Smurf2* siRNA transfected osteoblasts. Results are represented as fold changes compared to levels seen in scrambled siRNA transfected cells. (J) Analysis of *Rankl* expression in scrambled, *Smurf2* or *Synv1* siRNA transfected osteoblasts treated with vehicle or PTH (10nM) for 2 hours. Results are represented as fold changes compared to levels seen in vehicle treated scrambled siRNA transfected cells. (K) Co-immunoprecipitation assay in ROS17/2.8 cells showing an interaction between FLAG-Smurf2 and HA-HDAC4. Cell extracts were immunoprecipitated after 2 hours of treatment with PTH (10nM) or vehicle with an anti-FLAG antibody, proteins were detected by Western blot with either anti-HA or anti-FLAG antibodies. (L) Analysis of *Rankl* expression in WT or *Ppr+/-;Smurf2+/-* osteoblasts treated with vehicle or PTH (10nM) for 2 hours. Results are given as means ± SEM. *p<0.05 by Student's test.

Figure 3

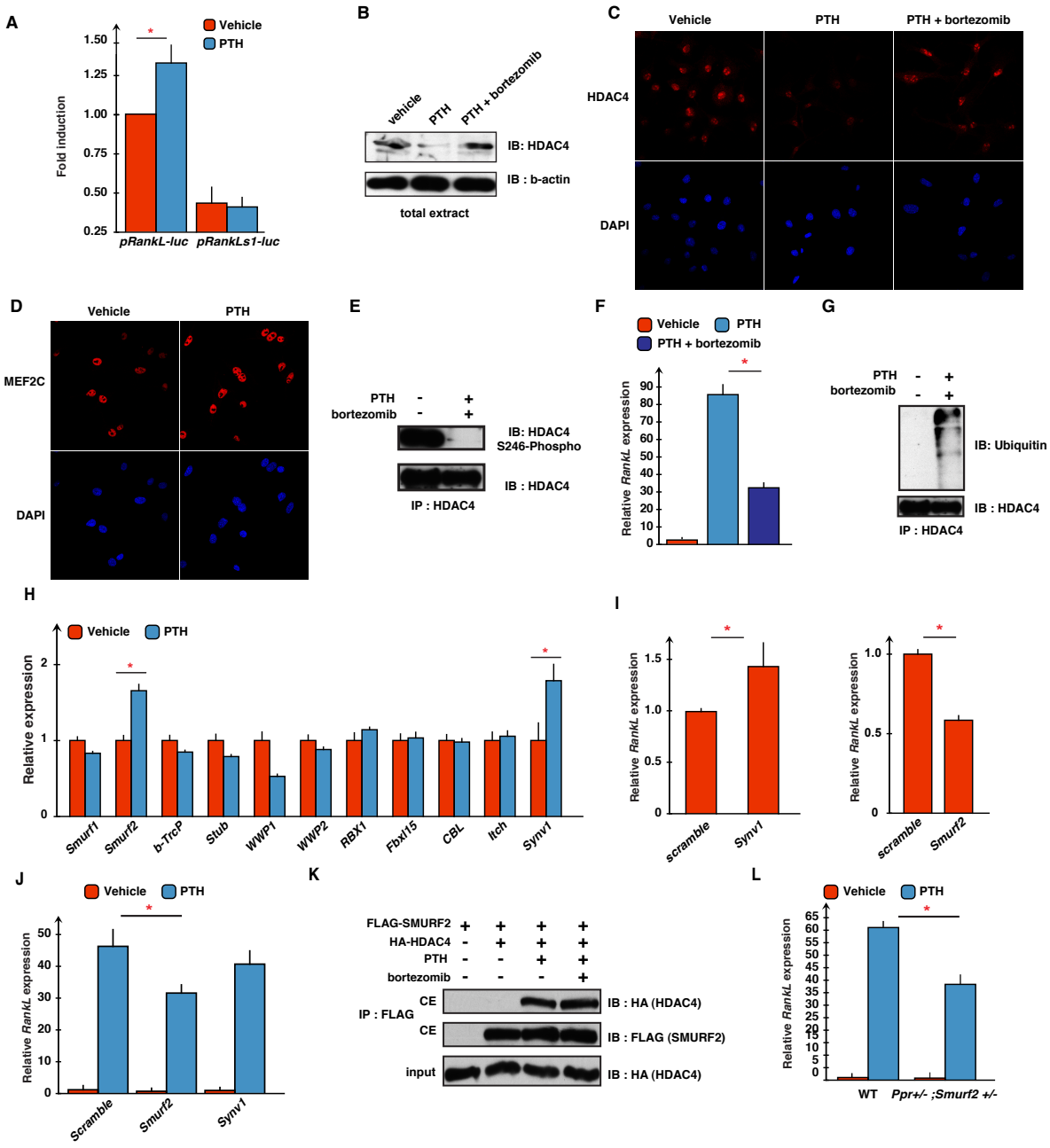

A
Vehicle
PTH
Fold induction
1.50
1.25
1.00
0.75
0.50
0.25
pRankL-luc
pRankLs1-luc
B
vehicle
PTH
PTH + bortezomib
IB: HDAC4
IB : b-actin
total extract
C
Vehicle
PTH
PTH + bortezomib
HDAC4
DAPI
D
Vehicle
PTH
MEF2C
DAPI
E
PTH
bortezomib
IB: HDAC4
S246-Phospho
IB : HDAC4
IP : HDAC4
F
Vehicle
PTH
PTH + bortezomib
Relative RankL expression
90
80
70
60
50
40
30
20
10
0
G
PTH
bortezomib
IB: Ubiquitin
IB : HDAC4
IP : HDAC4
H
Vehicle
PTH
Relative expression
2
1
0
Smurf1
Smurf2
b-TrcP
Stub
WWP1
WWP2
RBX1
Fbxl15
CBL
Itch
Synv1
I
Relative RankL expression
1.5
1.0
0.5
0
scramble
Synv1
1.0
0.5
0
scramble
Smurf2
J
Vehicle
PTH
Relative RankL expression
50
40
30
20
10
0
Scramble
Smurf2
Synv1
K
FLAG-SMURF2
HA-HDAC4
PTH
bortezomib
IP : FLAG
CE
CE
input
IB : HA (HDAC4)
IB : FLAG (SMURF2)
IB : HA (HDAC4)
L
Vehicle
PTH
Relative RankL expression
65
60
55
50
45
40
35
30
25
20
15
10
5
0
WT
Ppr+/- ;Smurf2 +/-

Figure 4. The sympathetic tone stabilizes HDAC4 to favor *Rankl* expression

(A) Analysis of the expression of *Hdac4* and *Hdac5* in WT osteoblasts treated with vehicle or ISO (10μM) for 2 hours. Results are presented as fold changes compared to levels seen in vehicle treated cells. (B) Immunofluorescence detection of HDAC4 in WT osteoblasts treated with vehicle or ISO (10μM) for 2 hours. (C) Co-immunoprecipitation assay in ROS17/2.8 cells showing an interaction between FLAG-ATF4 and HA-HDAC4/HA-HDAC5. Cell extracts were immunoprecipitated after 2 hours of ISO (10μM) treatment with an anti-FLAG antibody, proteins were detected by Western blot with either anti-HA or anti-FLAG antibodies. The numbers shown under each lane of the top blot represent the fold enhancement in the intensity of the bands. (D) Immunoprecipitation of HDAC4 in mouse osteoblasts treated with vehicle or ISO (10uM) for 2 hours. Proteins were analyzed by western Blot using either anti-HDAC4 or anti-HDAC4-S246P antibodies. (E) Serum CTX levels in 2 month-old control (n=8), $Hdac4_{osb}$+/- (n=3), $Atf4_{osb}$+/- (n=3), and $Atf4_{osb}$+/-; $Hdac4_{osb}$+/- (n=3) mice, shown as fold changes compared to levels seen in WT mice. (F) Analysis of *Rankl* expression in long bones of 2 month-old control (n=8), $Hdac4_{osb}$+/- (n=3), $Atf4_{osb}$+/- (n=3), and $Atf4_{osb}$+/-; $Hdac4_{osb}$+/- (n=3) mice. Results are presented as fold changes compared to levels seen in WT bones. (G) Histomorphometric quantification of the bone surface covered by osteoclasts in vertebrae of 2 month-old control (n=8), $Hdac4_{osb}$+/- (n=3), $Atf4_{osb}$+/- (n=3), and $Atf4_{osb}$+/-; $Hdac4_{osb}$+/- (n=3) mice. (H) Co-immunoprecipitation assay in ROS17/2.8 cells showing an interaction between FLAG-Runx2 and HA-HDAC4/HDAC5 (left panel). Cell extracts were immunoprecipitated after 4 hours of ISO (10μM) treatment with an anti-FLAG antibody; proteins were detected by western blot with either anti-HA or anti-FLAG antibodies. The numbers shown under each lane of the top blot represent the decrease in the intensity of the bands. Immunoprecipitation of Runx2 in mouse osteoblasts treated with vehicle or ISO (10uM) for 2 hours (right panel). Proteins were analyzed by western Blot using either anti-Runx2 or anti-HDAC4 antibodies. For all panels, results are given as means ± SEM. *$p<0.05$ by Student's test.

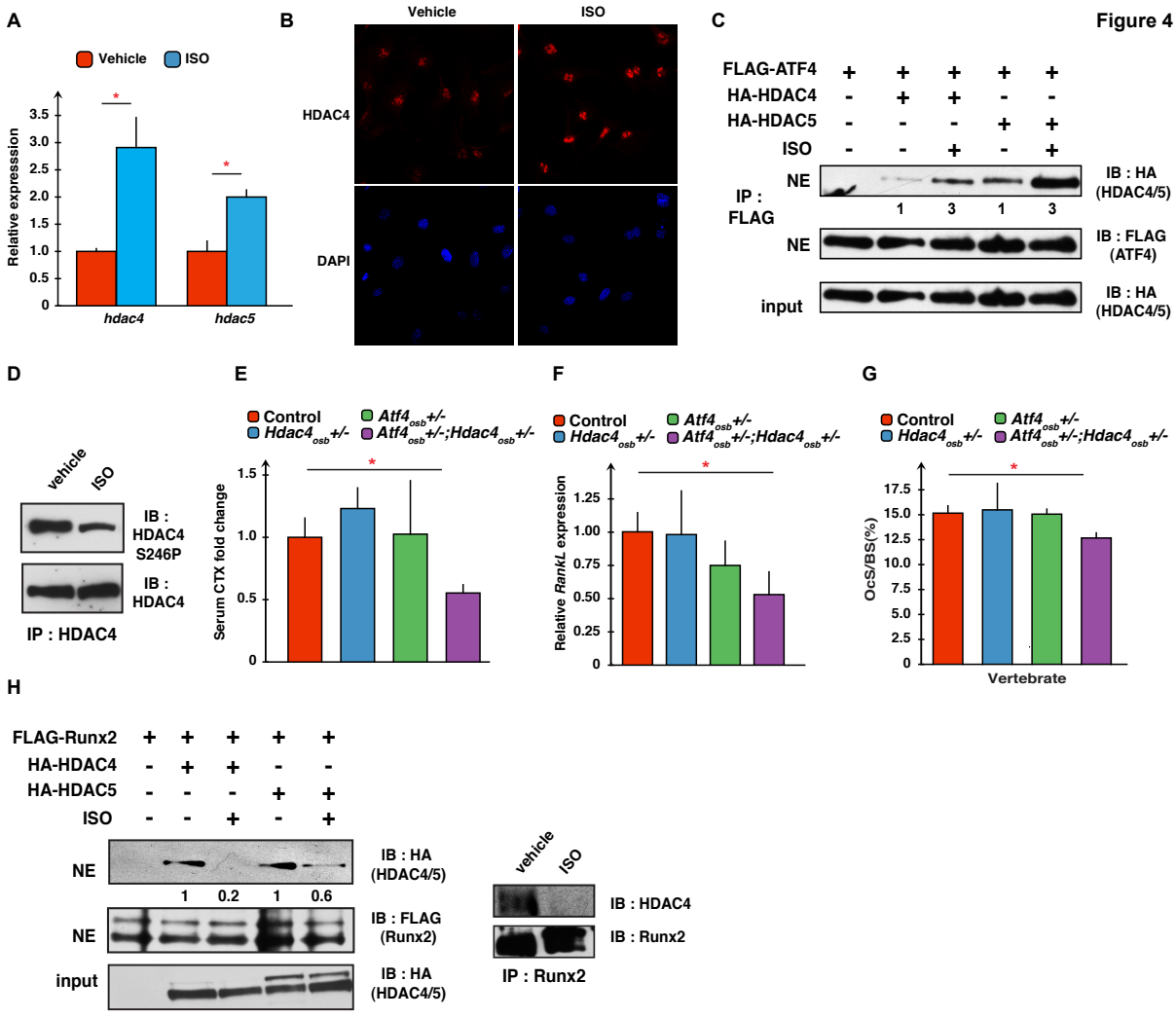

A
Vehicle
ISO
Relative expresssion
hdac4
hdac5
B
Vehicle
ISO
HDAC4
DAPI
C
Figure 4
FLAG-ATF4
HA-HDAC4
HA-HDAC5
ISO
NE
IP : FLAG
IB : HA (HDAC4/5)
IB : FLAG (ATF4)
input
D
vehicle
ISO
IB : HDAC4 S246P
IB : HDAC4
IP : HDAC4
E
Control
Hdac4osb+/-
Atf4osb+/-
Atf4osb+/-;Hdac4osb+/-
Serum CTX fold change
F
Relative RankL expression
G
OcS/BS(%)
Vertebrate
H
FLAG-Runx2
HA-HDAC4
HA-HDAC5
ISO
NE
IB : HA (HDAC4/5)
IB : FLAG (Runx2)
input
vehicle
ISO
IB : HDAC4
IB : Runx2
IP : Runx2

Figure 5. HDAC4 influences endocrine and cognitive functions of osteoblasts

(A) Analysis of expression of *Osteocalcin* and *Esp* in *Hdac4fl/fl* (n=11), *Hdac4$_{osb}$-/-*(n=11), control (n=8), *Atf4$_{osb}$+/-* (n=2), *Hdac4$_{osb}$+/-* (n=3), *Atf4$_{osb}$+/-;Hdac4$_{osb}$+/-* (n=4), WT (n=8) and *Hdac5-/-* (n=8) bones. Results are presented as fold changes compared to levels seen in bones of *Hdac4fl/fl* or WT mice. (B) Circulating levels of total and undercarboxylated osteocalcin in *Hdac4fl/fl* (n=11), *Hdac4$_{osb}$-/-*(n=11), control (n=8), *Atf4$_{osb}$+/-* (n=2), *Hdac4$_{osb}$+/-* (n=3), *Atf4$_{osb}$+/-;Hdac4$_{osb}$+/-* (n=4), WT (n=8) and *Hdac5-/-* (n=8) mice. (C) Serum insulin levels in *Hdac4fl/fl* (n=11), *Hdac4$_{osb}$-/-*(n=11), WT (n=8) and *Hdac5-/-* (n=8) mice. (D) Serum insulin levels in control (n=8), *Atf4$_{osb}$+/-* (n=2), *Hdac4$_{osb}$+/-* (n=3), *Atf4$_{osb}$+/-;Hdac4$_{osb}$+/-* (n=4) mice. (E) Glucose-stimulated insulin secretion (GSIS) test in 2 month-old *Hdac4fl/fl* (n=7) and *Hdac4$_{osb}$-/-* (n=6) mice. (F) Histomorphometric analysis of β-cell area (%) in pancreata of 2 month-old *Hdac4fl/fl* (n=7) and *Hdac4$_{osb}$-/-* (n=8) mice. (G) Insulin content in the pancreata of *Hdac4fl/fl* (n=7) and *Hdac4$_{osb}$-/-* (n=8) mice. (H-J) Testis size and weight, epididymes and seminal vesicle weights, sperm count and testosterone levels in *Hdac4$_{osb}$-/-* (n=5-8) and *Hdac4fl/fl* (n=5-9) mice. (K) Morris water maze test performed over 10 days. The graph shows the time (seconds) needed for *Hdac4fl/fl* (n=8) and *Hdac4$_{osb}$-/-* (n=9) mice to localize the platform in the swimming area. Results are given as means ± SEM. *p<0.05 by Student's test.

Figure 5

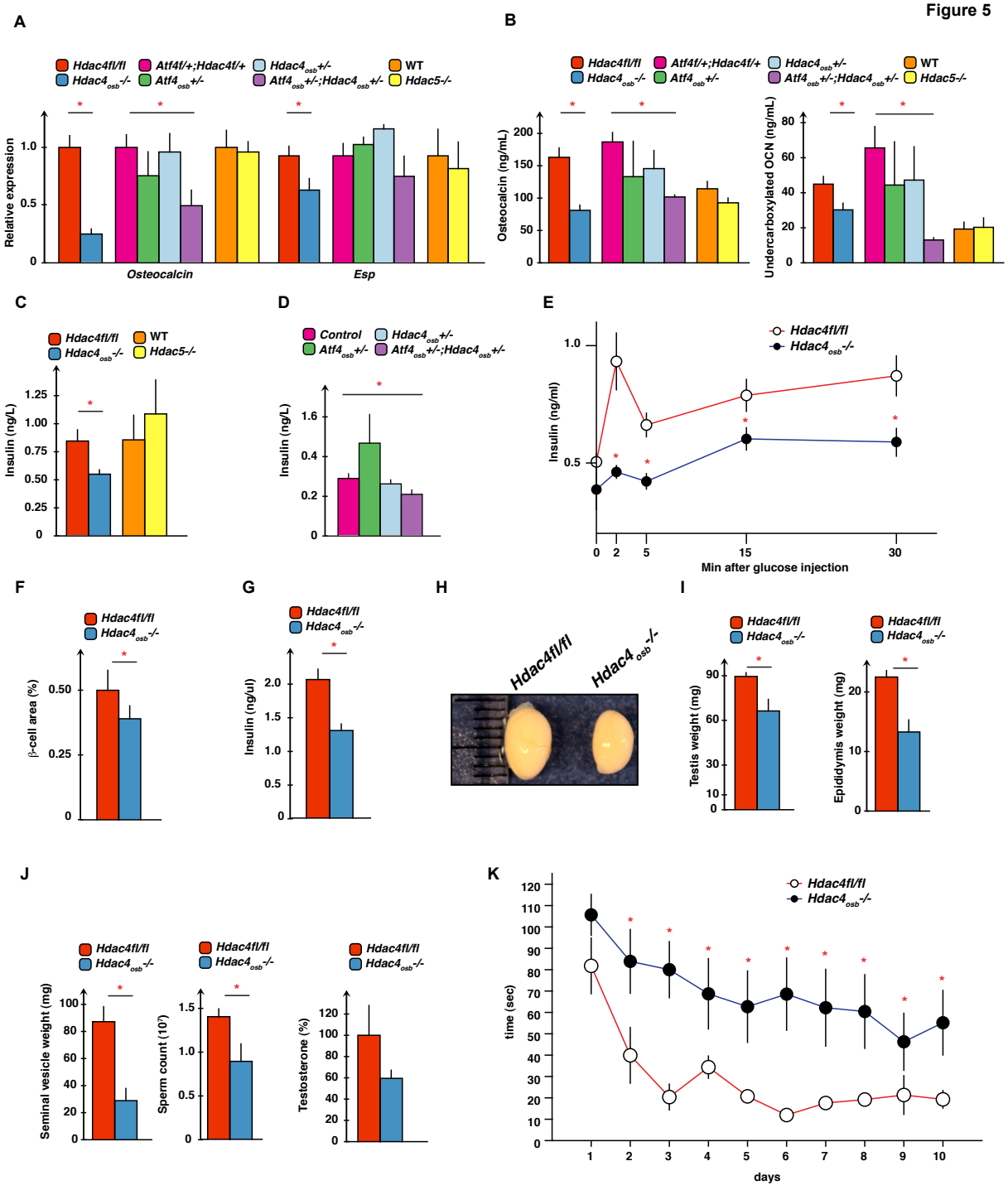

A
Hdac4fl/fl
Hdac4osb-/-
Atf4f/+;Hdac4f/+
Atf4osb+/-
Hdac4osb+/-
Atf4osb+/-;Hdac4osb+/-
WT
Hdac5-/-
Relative expression
Osteocalcin
Esp
B
Osteocalcin (ng/mL)
Undercarboxylated OCN (ng/mL)
C
Insulin (ng/L)
D
Control
Atf4osb+/-
Hdac4osb+/-;Hdac4osb+/-
E
Insulin (ng/ml)
Min after glucose injection
F
β-cell area (%)
G
Insulin (ng/ul)
H
Hdac4fl/fl
Hdac4osb-/-
I
Testis weight (mg)
Epididymis weight (mg)
J
Seminal vesicle weight (mg)
Sperm count (10⁷)
Testosterone (%)
K
time (sec)
days

Figure 6. Model of HDAC4 functions in osteoblasts

PTH signaling favors HDAC4 (in grey) degradation through polyubiquitination, this releases MEF2c that can now transactivate the *Rankl* promoter and favor bone resorption. On the other hand, following the sympathetic tone favors HDAC4 accumulation, its translocation to the nucleus and its association with ATF4; this favors *Rankl* expression and bone resorption as well as *Osteocalcin* expression and its endocrine and cognitive functions.

Figure 6

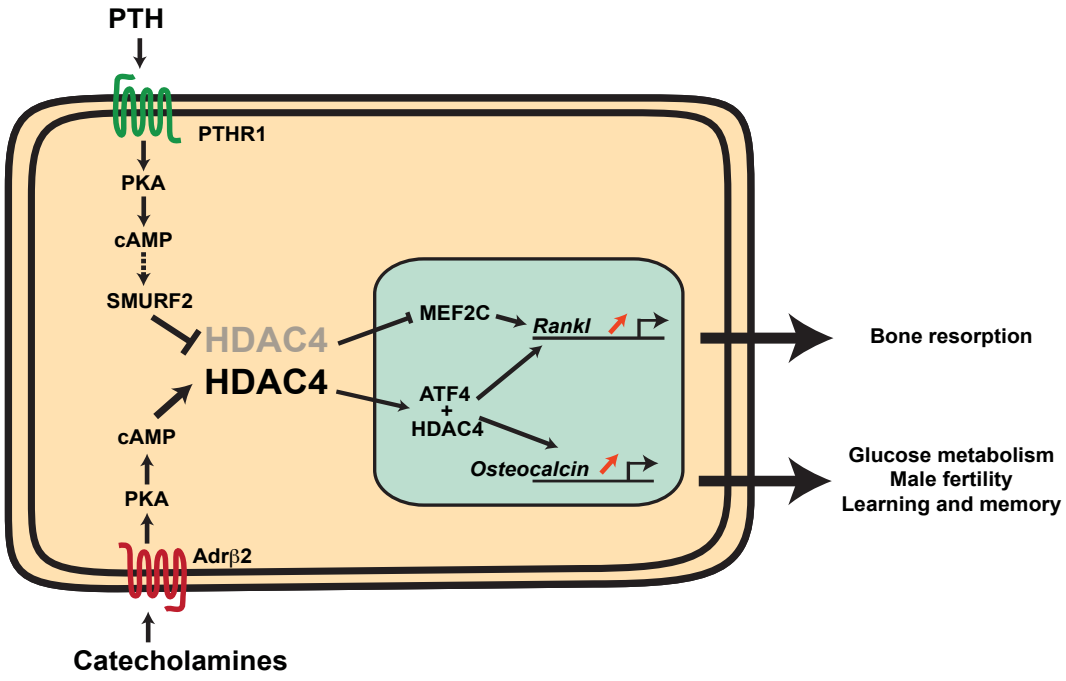


Figure S1. Related to Figure 1

(A) Detection of the deletion allele of *Hdac4* by PCR in genomic DNA isolated from tissues of *Hdac4*$_{osb}$*-/-*. PCR for GAPDH was used as a loading control. Analysis of the deletion efficiency of the *Hdac4* allele with the *Runx2-cre* transgene in *Hdac4*$_{osb}$*-/-* bone marrow osteoblasts. (B) Analysis of the expression of *Hdac4* in WT and *Hdac5-/-* bones (Left) and expression of *Hdac5* in *Hdac4fl/*fl and *Hdac4*$_{osb}$*-/-* bones. Results are represented as a fold change compared to levels seen in WT or *Hdac4fl/fl* bones. (C) Analysis of gene expression in *Hdac4fl/fl* (n=4), *Hdac4-/-* (n=4), WT, *Hdac5-/-* bones. Results are shown as fold changes compared to levels seen in *Hdac4fl/fl* bones. Results are given as means ± SEM. *p<0.05 by Student's test.

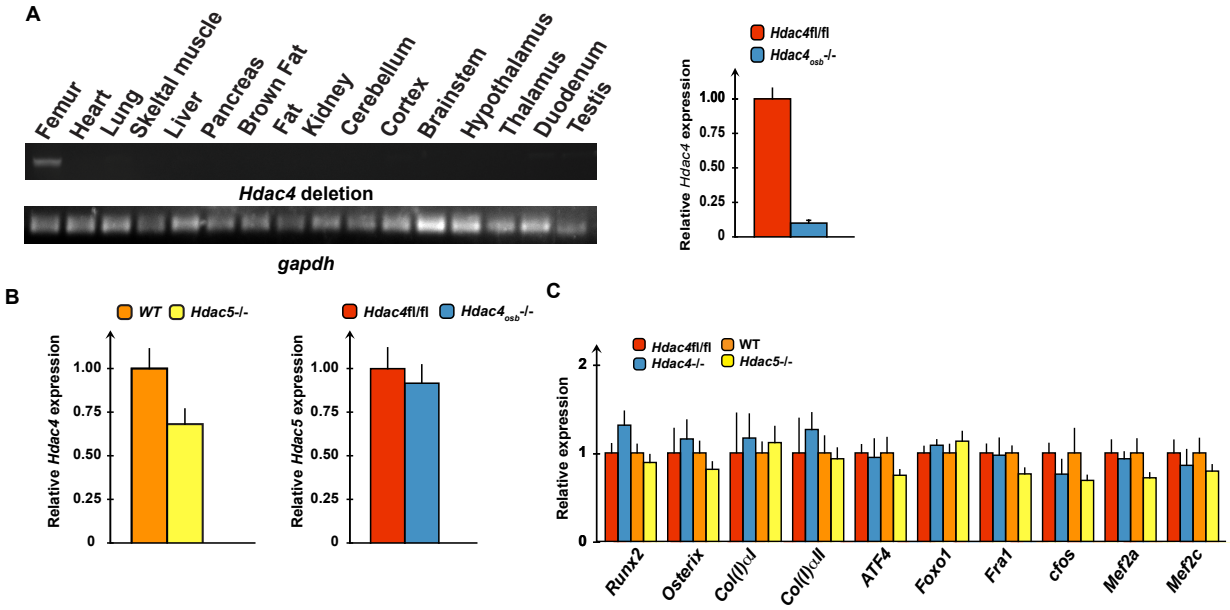


Figure S2. Related to Figure 2

(A) Detection of the deleted allele of *Mef2a* or *Mef2c* by PCR in genomic DNA isolated from tissues of *Mef2a*$_{osb}$*-/-* or *Mef2c*$_{osb}$*-/-* mice. PCR for GAPDH was used as a loading control. (B) Analysis of the deletion efficiency of the *Mef2a* or *Mef2c* alleles respectively with the *Runx2-cre* transgene in *Mef2a*$_{osb}$*-/-* or *Mef2c*$_{osb}$*-/-* bone marrow osteoblasts. Results are given as means ± SEM. *p<0.05 by Student's test.

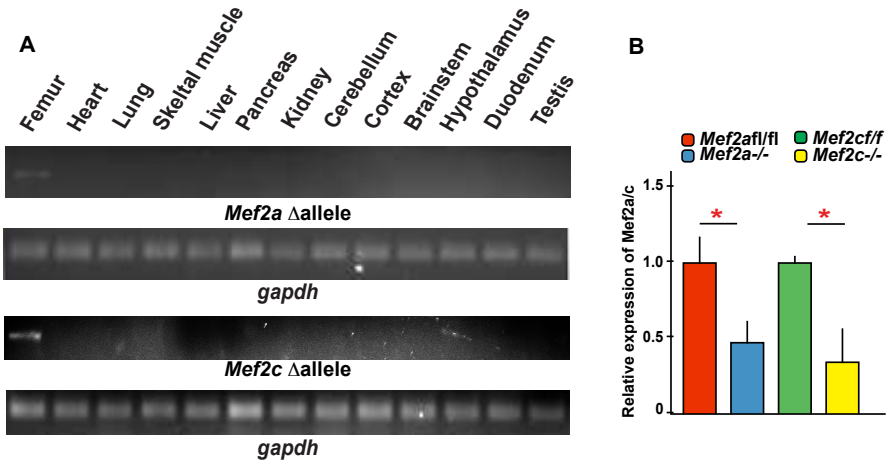


Figure S3. Related to Figure 3

(A) Analysis of the expression of *Hdac4* and *Hdac5* in vehicle or PTH (10nM) treated WT osteoblasts. Results are represented as a fold change compared to vehicle treated WT osteoblasts. (B) Analysis of the expression of E3 ubiquitin ligases in WT osteoblasts treated with ISO (10uM) for 2 hours. Results for each gene is presented as fold change compared to levels seen in vehicle treated osteoblasts. (C) Analysis of the expression of *Smurf2* and *Synv1* in primary osteoblasts transfected with either Smurf2 (left) or Synv1 (right) siRNA. Results are represented as a fold change compared to levels in scrambled siRNA transfected cells. (D) Analysis of *Hdac4* expression in scrambled, *Smurf2* or *Synv1* siRNA transfected osteoblasts treated with vehicle or PTH (10nM) for 2 hours. Results are represented as fold changes compared to levels seen in vehicle treated scrambled siRNA transfected cells. Results are given as means ± SEM. *p<0.05 by Student's test.

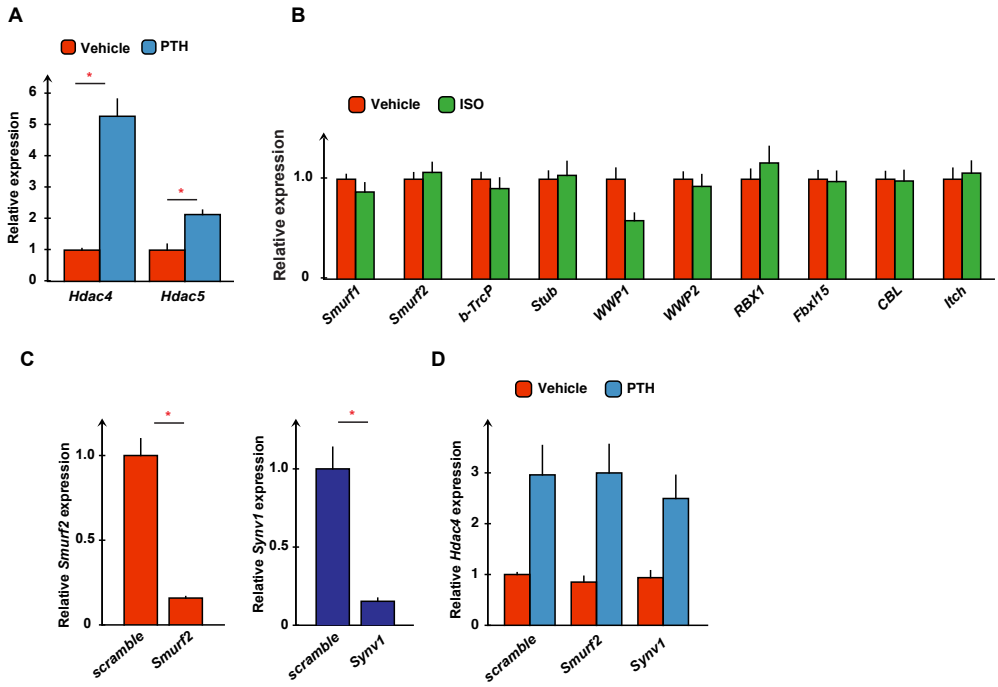
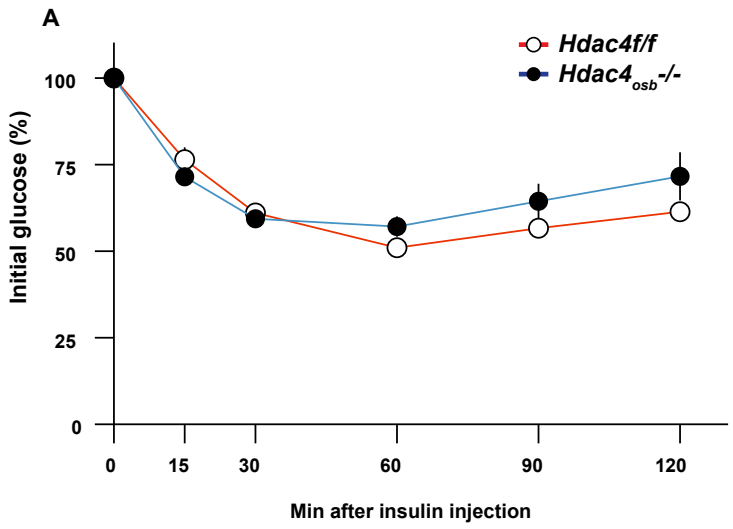


Figure S4. Related to Figure 5

(A) Insulin tolerance test (ITT) in 2 months-old *Hdac4fl/fl* and *Hdac4*$_{osb}$*-/-* mice. Results are given as means ± SEM. *p<0.05 by Student's test.



CHAPTER III. GENERAL DISCUSSION

Both PTH and sympathetic regulation of *Rankl* expression occur through a cAMP/PKA-dependent signaling cascade, for PTH through unidentified transcriptional means and for sympathetic signaling through ATF4 (Kondo et al. 2002; Elefteriou et al. 2005). Thus, this study was prompted by the question of how PTH and sympathetic signaling regulate *Rankl* expression through the same second messenger via different transcriptional means. We demonstrate here that HDAC4 is an integrator of these two signals in osteoblasts. On the one hand, we have shown molecularly and genetically that HDAC4 inhibits MEF2c function to transactivate *Rankl* promoter and PTH disrupts this inhibition by promoting HDAC4 polyubiquitination. On the other hand, sympathetic signaling promotes HDAC4 accumulation in the nucleus and its interaction with ATF4 to promote *Rankl* expression and thereby bone resorption as well as promoting osteoblast endocrine and cognitive functions by favoring osteocalcin expression and bioactivity (Figure 6).

The role of class II HDACs in regulation of *Rankl* expression in osteoblasts

Given that the overarching goal of this project was to understand how PTH and sympathetic signaling promote *Rankl* expression via different transcriptional means, we turned our attention to class II HDACs since this class of regulators has long been thought to connect the extracellular environment to the genome of a given cell (Verdin et al. 2003; Haberland et al. 2009). In addition, the regulation of Runx2 activity by HDAC4 during chondrocyte hypertrophy (Vega et al. 2004) also prompted us to ask whether Runx2 functions are also regulated by class II HDACs in osteoblasts. In order to elucidate the functions of class II HDACs in osteoblasts, we focused on HDAC4 and HDAC5 since they are the two most highly expressed members of this class of HDACs in osteoblasts. The analysis of the mice with the deletion of *Hdac4* only in osteoblasts and of mice lacking *Hdac5* revealed a novel function of these genes in bone remodeling. Unlike the expected high bone formation/high bone mass phenotype given that these HDACs were regulators of Runx2 in osteoblasts, we observed a high bone resorption/high *Rankl* phenotype in these mice. These results identified these 2 class II HDACs as inhibitors of bone resorption through inhibition of *Rankl* expression in osteoblasts.

Furthermore, our work on *Hdac4* and *5* led us to identify MEF2c as a major regulator of *Rankl* expression in osteoblasts. This finding is not surprising since MEF2 proteins are known to be involved in

multiple biological processes regulated by class II HDACs (Potthoff and Olson 2007; Haberland et al. 2009). There are three evidences that allowed us to identify MEF2c as a regulator of *Rankl* expression. First is the presence of three putative MEF2 binding sites within the *Rankl* promoter region that are conserved among various vertebrate species. Second, MEF2c transactivate *Rankl* promoter through its binding to these sites as mutating each of these sites abrogated the ability of MEF2c to transactivate the *Rankl* promoter and also decreased the basal activity of this promoter. Lastly, mice harboring a deletion of *Mef2c* specifically in osteoblasts have a significant decrease in *Rankl* expression and bone resorption.

In cell culture experiments both HDAC4 and HDAC5 hamper MEF2c ability to transactivate the *Rankl* promoter, while in vivo the functional interaction between HDAC4 and MEF2c was confirmed as $Hdac4_{osb}$*-/-* mice lacking one allele of *Mef2c* in osteoblasts had normal Rankl expression and bone resorption parameters. Furthermore, the analysis of *Hdac5-/-* mice with the deletion of one allele of *Mef2c* specifically in osteoblasts will also confirm genetically the regulation of MEF2c activation of *Rankl* expression by this class II HDAC, therefore also demonstrating in vivo, the importance of both of these class II HDACs in regulation of bone resorption through their expression in osteoblasts.

Identification of this novel function of HDAC4 and HDAC5 in osteoblasts to inhibit osteoclastogenesis adds a new depth to the knowledge of the transcriptional control of bone remodeling. Given that class II HDACs have been shown to have redundant functions in various tissues (McKinsey et al. 2000; Chang et al. 2004), and considering that mice with the deletion of *Hdac4* in osteoblasts and *Hdac5* globally display similar phenotype raises the question of whether the function of these two class II HDACs in regulating bone resorption could also be redundant. To answer this question, the analysis of the mice that lack *Hdac5* as well as *Hdac4* specifically in osteoblasts will elucidate further the functions of these two proteins in osteoblasts.

Although we defined the function of MEF2c during osteoclastogenesis, these findings still do not exclude the possibility that MEF2c might have other functions in osteoblasts that could be further pursued since we only focused on the *Rankl* expression and bone resorption phenotype of $Mef2c_{osb}$*-/-* mice. In fact, we noticed that bone formation was also affected in *Mef2c*-deficient mice, a phenotype we did not pursue in this study. A detailed analysis of the bone phenotype of $Mef2c_{osb}$*-/-* mice may also add to the

knowledge of the function of this family of transcription factors in bone formation and regulation of other functions of osteoblasts.

Another MEF2 family transcription factor that is highly expressed in osteoblasts is *Mef2a* that also transactivates *Rankl* promoter in vitro, however does not affect *Rankl* expression and bone resorption when deleted specifically in osteoblasts. This also raises the possibility that *Mef2a* might partially compensate for the functions of *Mef2c* in vivo. Analysis of mutant mouse line with the deletion of both of these genes only in osteoblasts will bring a broader knowledge to the functions of MEF2 family transcription factors in regulating osteoblast functions.

PTH regulation of *Rankl* expression in osteoblasts

PTH is one of the major endocrine signals regulating *Rankl* expression in osteoblasts. Although it was demonstrated through cell-based assays that CREB is the mediator of this signaling (Fu et al. 2002), deletion of *Creb* specifically in osteoblasts had no effect on *Rankl* expression or bone resorption parameters (Kajimura et al. 2011), thus leaving open the question of how PTH achieves this function in osteoblasts. The results presented above as well as the reported regulation of MEF2c functions in chick chondrocytes by PTHrP, a molecule showing sequence similarities with PTH (Kozhemyakina et al. 2009), prompted us to further test whether PTH is involved in the regulation of HDAC4 -| MEF2c → *Rankl* pathway.

PTH has a dramatic effect on HDAC4 as it decreases its accumulation in osteoblasts by promoting its ubiquitination and degradation. We further demonstrated that PTH-induced ubiquitination of HDAC4 occurs, in part, through *Smurf2,* one of the two E3 ligases among 11 that were tested, whose expression is enhanced upon PTH treatment in osteoblasts. Two other lines of evidence suggesting a role for Smurf2 in the PTH-induced ubiquitination of HDAC4 is the decrease observed in *Rankl* expression and its further induction with PTH in osteoblasts that lack *Smurf2* or one allele of *Ppr* and one allele of *Smurf2*.

We are aware, however, that further testing of Smurf2 in this pathway is required to confirm the functional relevance of this gene in vivo. In addition to the analysis of the mouse model lacking *Smurf2* specifically in osteoblasts, the analysis of *Rankl* expression in mice with deletion of one allele of *Ppr* and

one allele of *Smurf2* will strengthen the molecular data with genetic evidence to establish the validity of the proposed molecular pathway accounting for PTH regulation of *Rankl*.

Therefore with the work we present here, we do not only define MEF2c as the first transcription factor identified on genetic ground as a mediator of the PTH regulation of *Rankl* expression but also we further provide a mechanism for the regulation of the function of HDAC4 in bone resorption by PTH-induced ubiquitination in osteoblasts.

Regulation of *Rankl* expression by sympathetic signaling via HDAC4

The regulation of HDAC4 by PTH and the notion that class II HDACs connect various extracellular cues to the genome of a given cell prompted us to test whether sympathetic signaling, another endocrine regulator of *Rankl* expression (Elefteriou et al. 2005), might recruit HDAC4 for this purpose in osteoblasts. It was already known that the sympathetic tone regulates *Rankl* expression through ATF4 (Elefteriou et al. 2005). We show here that sympathetic signaling in osteoblasts achieves this function by enhancing *Hdac4* and to some extent *Hdac5* expression, promoting HDAC4 localization to the nucleus and enhancing HDAC4 and HDAC5 interaction with ATF4. We also demonstrate genetically that mice with a deletion of one allele of *Hdac4* and one allele of *Atf4* in osteoblasts display a low *Rankl*/low bone resorption phenotype.

Molecular data also suggests that the sympathetic regulation of the interaction between these two class II HDACs and ATF4 is specific to these two proteins. We demonstrate that the interaction between HDAC4/HDAC5 and Runx2 is disrupted by the sympathetic tone. Considering the constant presence of catecholamines in a living animal, this provides an explanation for why in the absence of HDAC4 or HDAC5, there is no effect on the Runx2-mediated bone formation.

These findings demonstrate that HDAC4 and ATF4 interaction promotes *Rankl* expression in osteoblasts and that this regulation is dependent on the sympathetic signaling. Further work is needed to confirm the validity of such a pathway in vivo. For this purpose, we have started the experiments to test the mouse model with deletion of one allele of *Adrenergic β_2 receptor (Adrβ_2)* and one allele of *Hdac4.* In the presence of such functional interaction, we expect that these mice will display a similar phenotype than *$Hdac4_{osb}$+/-;$Atf4_{osb}$+/-* mice, i.e., decreased *Rankl* expression and bone resorption parameters.

However, we also do not rule out the possibility that since PTH signaling will still be present in these mice, we may not observe a clear phenotype as demonstrated in *Hdac4*$_{osb}$*+/-;Atf4*$_{osb}$*+/-* mice. In that case, treating osteoblasts that lack one allele of *Hdac4* and one allele of *Atf4* with ISO and looking at *Rankl* expression will also be instrumental in demonstrating the sympathetic regulation of *Rankl* expression through ATF4 and HDAC4 interaction ex vivo. Given that sympathetic signaling is involved in such regulation of *Rankl* expression, we expect to see a significant decrease in the induction of *Rankl* expression in cells that lack one allele of *Hdac4* and one allele of *Atf4* compared to that in WT osteoblasts.

Further confirmation of the sympathetic regulation of HDAC4 will ensure that HDAC4 indeed is an integrator of PTH and sympathetic signaling in osteoblasts through its interaction with MEF2c for PTH and ATF4 for the sympathetic signaling. Although we have identified the transcriptional means of regulation of *Rankl* expression by these two extracellular signals in osteoblasts, further work with the use of elaborate proteomics is required to reveal the possible co-regulators that may also play a part in regulating the association of HDAC4 with either pathway. Identifying the molecular mechanism of action of these two signals in osteoblasts may provide means to develop more effective treatments against diseases characterized by increased bone resorption such as osteoporosis (Albright and Reifenstein 1948; Riggs et al. 1982).

Sympathetic regulation of endocrine functions of bone through HDAC4

Over the last decade, bone has been shown to be an endocrine organ regulating glucose homeostasis, promoting male fertility as well as memory and cognition through the synthesis and secretion of the hormone osteocalcin (Karsenty and Ferron ; Oury et al. 2011; Oury et al. 2013b).

The interaction of ATF4 with HDAC4 and HDAC5, and the regulation of *Osteocalcin* expression by ATF4 (Ducy and Karsenty 1995; Yang et al. 2004) raised the question of whether osteocalcin functions might be regulated through the same mechanism. In addition to the dramatic decrease in osteocalcin expression in *Hdac4*$_{osb}$*-/-* mice, the decrease in the circulating levels of total and undercarboxylated osteocalcin prompted us to further pursue the phenotype of these mice in more detail.

Indeed, *Hdac4*$_{osb}$*-/-* mice display similar defects than those observed in *Osteocalcin+/-* mice in insulin secretion and circulating insulin levels, male fertility and memory and spatial learning (Lee et al. 2007; Oury et al. 2011; Oury et al. 2013b). That deletion of *Hdac5* in mice does not affect *Osteocalcin* expression or its circulating levels suggests that this function of HDAC4 is specific to this class II HDAC and that the existence of a specific co-factor associated with HDAC4 but not HDAC5. Thus, proteomic and biochemical studies that will need to be used to identify the co-factors associated with HDAC4 downstream of PTH and sympathetic signaling will also be useful in identifying the different factors that promotes this specific function of HDAC4 in regulating osteoblast endocrine and cognitive functions. These data altogether suggest that HDAC4 is central to the regulation of osteocalcin functions, demonstrating the emerging role of osteoblasts as a multipurpose cell type (Karsenty and Ferron 2012).

However further confirmation of the regulation of osteocalcin functions through sympathetic signaling is needed. Therefore, further experiments are required to assess whether through its regulation of the interaction between HDAC4 and ATF4, sympathetic signaling is a regulator of osteocalcin functions. To answer this question, we will test the functional interaction between HDAC4 and sympathetic signaling in osteoblasts with the use of mouse genetics. Answering this question will not only identify a direct link between sympathetic tone and molecular mechanism of regulation of osteocalcin functions but will also further add to the existence of the cross-talk between the bone and the whole body, defining a second level regulation of *Osteocalcin* expression and its neuroendocrine functions.

Finally, these findings also suggest that HDAC4 may be involved in some aspects of aging. The abnormalities observed in the absence of osteocalcin with worsened glucose metabolism, decreased male fertility, decreased cognition and memory and the decrease in bone mass usually appear as age-related issues. HDAC4 regulation of these functions presented here suggests a role for this class of regulators during aging process and encourages a new way of looking at their regulation and involvement in various signaling pathways.

CONCLUSION

Overall, this work showed that the molecular means of PTH regulation of *Rankl* expression occurs through the regulation of MEF2c transactivating function and that PTH achieves this function by promoting ubiquitination of HDAC4, in part, via Smurf2. Furthermore, this work provides evidence that HDAC4 is indeed an integrator of PTH and sympathetic signaling in the osteoblast. In addition, the completion of the suggested experiments that will strengthen the findings of this study will prove again that bone is central to the whole body physiology as an endocrine organ by demonstrating a direct link between sympathetic signaling and osteocalcin functions via Class II HDAC (Figure 6).

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